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THE UNIVERSITY OF ALBERTA

The interactions between Glossina morsitans and host rabbits: A host/biting-fly model

by

©

Kenneth Robert Parker

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

Department of Entomology

Edmonton, Alberta

Spring, 1978

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled The interactions between *Glossina morsitans* and host rabbits: A host/biting-fly model.

submitted by Kenneth Robert Parker

in partial fulfilment of the requirements for the degree of Master of Science.

R. H. Gooding
Supervisor

B. K. Mitchell

Date December 19, 1977

This thesis is dedicated
to my mother, Joan.

We dance round in a ring and suppose,
But the Secret sits in the middle and knows.

-Robert Frost-

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ABSTRACT

Glossina morsitans morsitans (Westw.) were maintained on rabbits. Tsetse salivary glands were examined for properties important to host-ectoparasite interaction. The anticoagulant in the salivary glands was demonstrated as an antithrombin which inhibits both thrombin's esterolytic and proteolytic activity. It is distinct from both heparin and hirudin and is a single, probably non-proteinaceous substance with a molecular weight of 11,000 to 13,000. Salivary gland homogenates contained no haemolytic or fibrinolytic activity, and did not lose anticoagulant activity when stored for one month at +4° or -20°C, or upon heating at 98°C for 60 minutes. The presence of such homogenate in platelet-rich human plasma inhibited aggregation induced by various aggregating agents. Inhibition of platelet aggregation and release are suggested to be a result of the antithrombin's action.

Rabbits exposed to tsetses developed precipitating antibodies to tsetse salivary gland homogenate. Sephadex fractions of the homogenate containing maximum anticoagulant activity produced no precipitin reaction when incubated with rabbit sera containing antibodies to tsetse salivary gland homogenate. Similarly, anticoagulant activity was not diminished when incubated with rabbit antisera. Whole blood and plasma clotting times of exposed rabbits were normal.

Host-rabbit weights and haematological parameters were not affected with exposures of 250-500 flies per day, 2 to 3 days a week. The same exposure 6 days a week resulted in host anemia, but no consistent loss of weight. Some rabbits gained weight, indicating that a salivary toxin may be affecting host-metabolism. Host-anemia correlated negatively with fly exposure. Fly blood-meal weights, used as a measure of local resistance, proved unsatisfactory for such purpose. However, males took consistently smaller meals than females, and backs of rabbits were less favourable for feeding than ears. Fly probings, counted during feeding, produced similar results. A ratio of fly meal weight over the number of probes required to obtain a blood meal was used as a measure of feeding efficiency. Arteriolar vasoconstriction, observed in rabbits following heavy tsetse exposure, is believed to play a major role in host-resistance. Antibody titres were quantitatively determined using passive haemagglutination, and found to correlate, to some extent, with tsetse exposure.

Experiments demonstrated naturally acquired host-resistance affects the tsetse. Pupal weights were most sensitive, followed by female survivorship, female productivity, and male survivorship respectively. Emergence of both sexes from pupae was never affected. Resistance was demonstrated as local. Rabbits acquire different levels of resistance. In experiments designed to determine if naturally produced antibodies are detrimental to the tsetse, fly parameters were not affected. The role of these antibodies remains unknown. Host-anemia, caused by excessive tsetse feeding, was shown to reduce the pupal weights of flies maintained on anemic rabbits.

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CHAPTER 1

INTRODUCTION

1.1 Current status of research on host-ectoparasite relationships

In the context of this thesis, the following definitions have been adopted. An ectoparasitic arthropod is an arthropod which is closely associated with at least one vertebrate host for all or a part of its life. A host-ectoparasite relationship refers to the physiological interaction between host and parasite (review by Nelson et al., 1975). Under natural conditions, host-ectoparasite interaction is influenced by many factors including fluctuations in host and parasite densities, changes in host nutritional and physiological status, environment and season. However, laboratory conditions may eliminate or control such factors, permit interaction with fewer variables, and thus reduce the possibility of misinterpretation. Unfortunately however, there are disadvantages to using laboratory models. There are arguments that the artificiality of association between host and ectoparasite does not approximate interaction under natural conditions. Second, only a limited number of arthropods have been successfully cultured, restricting the choice of ectoparasite. Third, high costs are involved in maintaining statistically adequate numbers of hosts and parasites (review by Nelson et al., 1975).

The advantages of laboratory conditions are more numerous, and arthropods offer advantages over internal parasites for controlled studies, since they allow direct observation, manipulation, and enumeration (review by Nelson et al., 1975). Aside from controlling those factors which fluctuate under natural conditions, laboratory research permits selective ectoparasite exposure. Under the appropriate conditions, intraspecific ectoparasite competition may also be controlled, eliminating a factor unsuitable for monitoring ectoparasite responses correlating with resistance. Certain conditions prevailing, higher levels of host exposure may also be achieved over a shorter period of time. Although the choice of ectoparasite is limited, those species which have been successfully cultured provide statistics essential for evaluating levels of acquired host-resistance. Complications of disease transmission and competition with internal parasites may also be eliminated.

In a recent review, Steelman (1976) points out that those authors who studied host-ectoparasite relationships under laboratory conditions dealt mainly with the role of disease transmission rather than with changes in the host's physiology. He also points out that adequate information on host-ectoparasite relationships is essential, but unfortunately not available, for determining which levels of arthropod infestation warrant control measures.

The feeding behavior of blood-sucking arthropods has been characterized as either slow or rapid (Tatchell, 1969). While research has emphasized the slow feeders such as the ticks because they are easily manipulated, comparable research with biting-flies is lacking. Furthermore, results of studies should be replicated to account for the variability of response in host and parasite. Integrating the phenomena associated with host-ectoparasite relationships involves studies from a number of related fields including physiology, immunology, biochemistry, haematology, entomology and ecology. A restricted number of researchers, limited finances, and as pointed out by Nelson et al. (1975), the fact that those persons who are involved are reluctant to study problems in a field outside their own, explain the paucity of information in this field.

1.2 Choice of host and parasite

Choice of uninfected Glossina morsitans morsitans (Westw.) as the ectoparasite, and Flemish Giant x French Lop-eared rabbits as hosts, creates an artificial association which does not occur in nature. Despite this artificiality, this particular choice of host and parasite offer advantages over most other laboratory models.

Unlike the Nematocera, both sexes of the tsetse are obligatory blood feeders, allowing all specimens to be used in experiments. Tsetses take among the largest blood meals of the blood-sucking flies, and are second only to the tabanids in taking the largest blood meal of the biting-flies (review by Gooding, 1972a). Furthermore, tsetses feed more frequently and live longer than most flies, allowing a greater amount of blood obtained per insect. From an economical standpoint, this might allow fewer tsetses to be cultured than might be required to produce a similar host response with an alternative parasite. It was also hoped that changes in host physiology might be more readily detectable. Fortunately, the tsetse has been successfully cultured for about a decade, providing well established population statistics. Present maintenance techniques allow flies to be handled with ease and exposure to be selectively controlled. The tsetse is a particularly good choice for studying parameters affected by host-resistance. Blood is their sole source of nourishment. The reproductive cycle provides regular production of pupae which are large enough to handle singly and to weigh with considerable accuracy. Tsetse longevity allows prolonged experimentation with the same flies. Recent detailed accounts of tsetse digestive enzymes and their role in breaking down the blood meal (Gooding, 1974a, 1974b, 1977; Gooding and Rolseth, 1976) may also provide information to aid in elucidating the mechanism of host-resistance.

The piercing and sucking mouthparts of the tsetse permit repeated feeding on the same host, or even the same part of the host without serious damage; a procedure which might prove difficult with tabanids or blackflies. The size and robustness of the tsetse flies permits repeated handling without serious deleterious effects; this might prove difficult with mosquitoes. Glossina, like many other blood feeding flies, contain a powerful anticoagulant (Yorke and MacFie, 1924; Lester and Lloyd, 1928; Hawkins, 1966). The tsetse anticoagulant, being more powerful than most, makes it more suitable for study.

Host rabbits are small enough to maintain in controlled environments, although sufficiently large to feed 60 flies in 15 minutes. Since these rabbits have been maintained in a closed colony for several years, and since control and experimental rabbits may be drawn from one litter, genetic variability could also be controlled. Rabbits are also known to be immunologically responsive.

1.3 Application of the proposed model

Causing losses of 5 billion dollars annually, and limiting use of 4.5 million square miles of Africa by man and his domestic animals, the tsetse is the most economically damaging arthropod (review by Steelman, 1976). North America has no comparable counterpart. The purpose of this research therefore, was to provide information to those working with ectoparasites, and specifically to those concerned with tsetse husbandry.

Although the tsetse has been successfully maintained in culture for about 10 years, there are occasional reports of decreases in colony production for no apparent reason (review by Laird, 1977). Although subtle factors such as feeding frequency (Langley and Pimley, 1975) and toxicants in host diets (Saunders, 1971; Jordan and Trewern, 1976; review by Laird, 1977) may account for some of these fluctuations in productivity, others remain unexplained. Another possibility which has received little attention, is the influence of acquired host-resistance resulting from repeated exposure to tsetses. Apparently only two occurrences of cutaneous responses, both with goats, have been reported (Nash et al., 1965; Nash, 1970). Tsetse researchers have partially eliminated the problem through host exposure at an early age and exchanging hosts at regular intervals. Many colonies are increasing productivity by switching to hosts such as rabbits or guinea pigs (review by Laird, 1977). However, there appear to be no reports of rabbit or guinea pig responses to tsetse exposure. There are two benefits to studying this problem. First, diminishing acquired host-resistance may allow an increase in colony productivity, providing a larger number of expendable specimens from a self-sustaining colony for research. An increase in colony productivity means a smaller colony is required to produce the same number of expendable specimens, reducing maintenance, and subsequently costs. Second, improving conditions in cultures may provide a physiologically optimal tsetse. These specimens would be more advantageous for use in male sterility release programs than males produced by regimes presently in use, since they would better compete with wild tsetses. Presumably, these specimens would also be superior for use in physiological experiments.

The second purpose of this research was to examine and test literature reports of host-ectoparasite interaction with a laboratory model. It was also hoped that this study would provide some results which would serve as a guideline for pursuing host-ectoparasite interaction with biting-flies under natural conditions. Carefully controlled conditions are necessary to detect subtle physiological changes. Understanding these subtle changes may be essential in interpreting and understanding host-ectoparasite relationships, even though such changes may be undetectable under natural conditions. At present, much of the research on host-ectoparasite interaction emphasizes the development, detection, and mechanisms underlying host-resistance. Attempts are being made to determine if resistance is innate or acquired, local or systemic, and whether it is mediated through cutaneous responses, biochemical phenomena, cell infiltration, antibodies, genetically, or a combination of two or more. Two excellent, recent, and comprehensive reviews by Nelson et al. (1975, 1977) will acquaint the reader with the present status of research in this field.

1.4 Objectives

This research was designed to examine 3 aspects of host-ectoparasite interaction. Because a number of changes occur among hosts and parasites simultaneously, 3 aspects were examined rather than one in greater detail. Initial hopes were to integrate changes in one aspect with the others. The objectives were:

1. to determine those properties of the tsetse saliva which may play an important role in host-ectoparasite interaction, specifically the effect of the salivary anticoagulant on haemostasis.
2. to determine the physiological responses of host-rabbits to

tsetse exposure, specifically haematological, serological, and cutaneous reactions which either result from, or influence the blood meals obtained by the tsetse.

3. to determine the effects of naturally acquired localized resistance and circulating antibodies upon the tsetse, specifically adult longevity, pupal production, pupal weights, and emergence from pupae.

The relative importance of each objective is discussed in its respective chapter.

1.5 Colony history and maintenance

The colony of uninfected Glossina morsitans morsitans (Westw.) originated from approximately 1,000 pupae obtained from the Tsetse Research Laboratory, University of Bristol, England, in July, 1973. Since that time a closed colony of 1,000 to 2,000 flies, most of which were productive females, has been maintained in the Department of Entomology, University of Alberta, on rabbits originating from a cross of Flemish Giant x French Lop-eared rabbits. Maintenance procedures were those described by Nash et al. (1971) with a few modifications. The colony was maintained at 21° to 24°C under moderate relative humidity. Flies were kept 15 to a Geigy-10 cage (16.5 x 8.5 x 5 cm), and placed on the ears or clipped backs of rabbits for 15 - 20 minutes each day, 6 days a week. The diameter of the terylene mesh netting used for the cages permitted flies to feed directly on hosts when placed on the back or ears, as well as permitting larvae to pass out of the cages to pupate in plexiglass boxes layered with 1-2 cm of sand (Fig. 1). Rabbits were restrained in holding boxes equipped with neck collar and ear platforms (Nash et al., 1971). Four Geigy-10 cages, one on each ear, and two on the back, were strapped to rabbits with

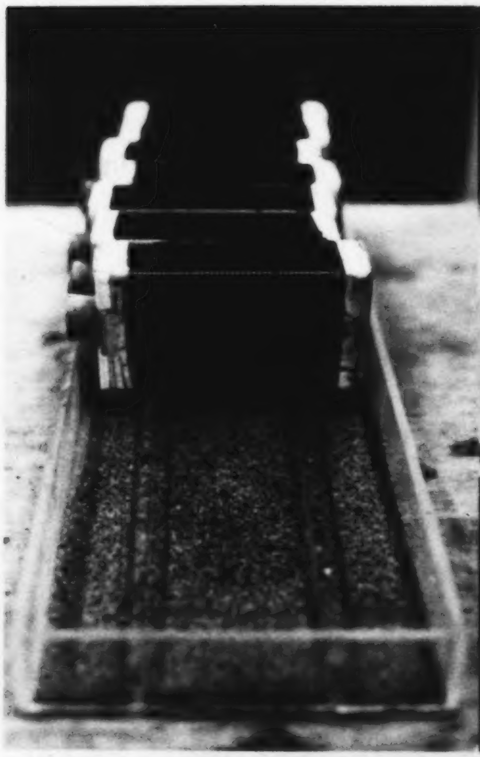


Figure 1.

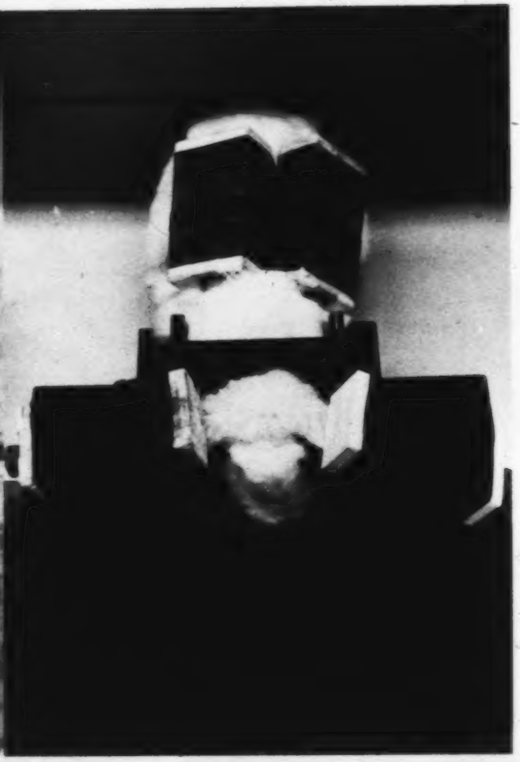


Figure 2.

Figure 1. Technique used to collect pupae produced by tsetses.

Figure 2. Rabbit restrained in holding box while under exposure to tsetses. A felt sheet is normally placed over the rabbit to reduce disturbance while flies are feeding.

elastic and changed every 15 or 20 minutes (Fig. 2). Use of the restraining box permitted flies to feed without interference from host-grooming or excessive movement. Using a limited number of flies per cage reduced intraspecific competition between flies while feeding and at other times. Schematic representation of the tsetse life cycle in relation to colony maintenance is shown in Appendix A, Fig. A1.

Rabbits were maintained by Bioscience Animal Services, University of Alberta. Rabbits were used from age 6 months and exposed, on average 2 - 3 times per week and at most 6 times a week, for a period usually less than 5 hours on any one day. Some rabbits were used over two and one-half years. Rabbits were maintained at 17.7±1.3°C on a 15-9 hour day-night cycle and fed a standard lab diet of Master Baby rabbit pellets (Maple Leaf Mills, Edmonton) containing no antibiotics or coccidiostats. These additives were known to effect tsetse productivity (Saunders, 1971; Jordan and Trewern, 1976). Because of the importance of host nutrition in suppressing the effects of arthropods (see review by Nelson et al., 1975), composition and estimated weekly nutritional intake of this diet are provided (Appendix A, Table A1). Food and water were available ad libitum.

CHAPTER 2

EFFECT OF Glossina morsitans SALIVARY GLAND HOMOGENATE ON HAEMOSTASIS

List of abbreviations for Chapter 2

ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
APTT	Kaolin-activated partial thromboplastin time
AtIII	Antithrombin III
CNP	Citrated normal plasma (commercial), used for test check
CPP	Citrated pooled plasma, 9 parts whole blood to 1 part 3.8% sodium citrate and centrifuged
CPP/AtIII deficient	Citrated pooled plasma titrated free of Antithrombin III activity by addition of anti-AtIII serum
EDTA	Na_2 = disodium-, K_3 = tripotassium-, ethylenediamine tetraacetate
NaCl/CPP	1 part NaCl to 6 parts CPP, unless otherwise stated
NaCl/PRP	1 part NaCl to 9 parts PRP, platelet count adjusted to $3.0 \times 10^5/\mu l$ for platelet adherence to collagen and $2.0 \times 10^5/\mu l$ for platelet aggregation studies
PPP	Citrated platelet-poor plasma, same proportions as CPP
PRP	Citrated platelet-rich plasma, same proportions as CPP
PT	Prothrombin time
SEM	Scanning electron microscopy
SGS	Glossina morsitans morsitans salivary gland solution, 100 pair of salivary glands/ml of NaCl, unless otherwise stated

SGS/CPP	1 part SGS to 6 parts CPP, unless otherwise stated
SGS/PRP	1 part SGS to 9 parts PRP, platelet count same as NaCl/PRP
TAME	p-tosyl-L-arginine methyl ester
TT	Thrombin Time

2.1 Introduction

The salivary glands of haematophagous insects often harbour causative organisms of disease, secretions which result in allergic and immune responses, and toxins which have a direct effect on the host's physiology. Knowledge of the properties and mechanisms of action of salivary components is important to an understanding of host-ectoparasite relationships.

Saliva plays a major role in preventing premature clotting of blood in the mouthparts during feeding and while stored in the crop (Lloyd, 1928; Lester and Lloyd, 1928; Hudson et al., 1960; Hudson, 1964). Shortly after feeding, the ingested blood volume is substantially reduced through defecation of water from the serum (Boorman, 1960). Consequently, the properties most frequently studied in the saliva or salivary gland emulsions of blood-sucking insects are anticoagulants and agglutinins (review by Hellmann, 1968; review by Gooding, 1972a; Maretic and Zekic, 1973; Yang and Davies, 1974). A powerful anticoagulant occurs in the salivary glands of Glossina spp. (Yorke and MacFie, 1924; Lester and Lloyd, 1928; Hawkins, 1966). The anticoagulant in the salivary glands of G. morsitans was first described as inhibiting formation of thrombin (Lester and Lloyd, 1928). More recently, antithrombin activity has been reported in the salivary glands of G. austeni (Hawkins, 1966) and from results of histochemical analysis of its saliva, Fairbairn and Williamson (1956) postulate the anticoagulant is of low molecular weight, probably neither heparin nor an enzyme. Antithrombins have also been reported in the extracts of Hirudo medicinalis (hirudin: Markwardt, 1957), the salivary glands of Tabanus bovinus (tabanin: Markwardt and Leberecht, 1959), the gut of saline fed Rhodnius prolixus (reduviin: Markwardt and Shulz, 1960), and in the salivary glands, gut, eggs, and coxal fluid of Ornithodorus moubata (Hellmann and Hawkins, 1967). Hirudin and reduviin, but not tabanin, inhibit thrombin hydrolysis of TAME (Markwardt, 1961), indicating inhibition of the esterolytic as well as the proteolytic site on the thrombin molecule (Seegers, 1967).

Coagulation inhibitors, other than antithrombins, occur in blood-sucking arthropods. The anticoagulant from the gut of saline fed O. moubata inhibits thrombokinase (Markwardt and Landmann, 1961), and in addition to antithrombin activity, Hellmann and Hawkins (1967) described anti-factor IX activity in the salivary glands. Anti-factor VIII activity has been demonstrated in the salivary glands of Rhodnius prolixus, as well as a second anticoagulant with antithrombin activity, which occurs in the gut 4 hours after feeding (Hellmann and Hawkins, 1965). Unidentified anticoagulants, which are not antithrombins, have been described in the salivary glands of Stomoxys calcitrans, various species of mosquitoes (Hudson, 1964), and Triatoma maculata (Hellmann and Hawkins, 1966).

The presence of fibrinolytic activity in the salivary glands of haematophagous insects suggests a possible role in blood digestion and a mechanism for destruction of small clots in canals of the mouthparts. Plasminogen activators are reported in the salivary glands of Glossina austeni (Hawkins, 1966) and the coxal glands but not the salivary glands of Ornithodorus moubata (Hellmann and Hawkins, 1967). Fibrinolytic activity has also been demonstrated in the gut tissue, but not the salivary gland emulsions, of Rhodnius prolixus and Triatoma maculata (Hellmann and Hawkins, 1966).

A digestive role of salivary secretions is further substantiated by reports of haemolytic properties. In a review by Nelson et al. (1975), Dem'Yanchenko (1960) is cited as demonstrating a haemolytic toxin in the thoracic parts, including the salivary glands of Simuliids. Saliva of Cimex lectularius hemolyzed some, but not all types of erythrocytes (Sangiorgi and Frosini, 1940). Salivary gland emulsions of Culex pipiens, C. annulata, Aedes aegypti, Glossina tachinoides, and Phlebotomus papatasi have no haemolytic properties (review by Gooding, 1972a).

The digestive enzymes in the salivary glands of haematophagous insects have been reviewed (Gooding, 1972a, 1975; Wigglesworth, 1972). Geczy et al. (1971) suggest that esterases in the salivary glands of Boophilus microplus may increase vascular permeability in the skin of sensitized hosts. Prostagladin-E_2 in the saliva of this species may play a role in the initiation and maintenance of the lesion of the host (Higgs et al., 1976). Histamine-like substances which affect vasodilation and immune responses have been demonstrated in Culex pipiens (Eckert et al., 1951), Simulium sp. (Hutcheon and Chivers-Wilson, 1953), and Aedes stimulans (Wilson and Clements, 1965). Substances similar to bradykinin have also been described (review by Nelson et al., 1977).

Arthropod secretions contain toxins which directly affect host physiology. Tick paralysis and the effects of some insect toxins have been reviewed (Beard, 1963; Zlotkin, 1973; Gregson, 1973; Nelson et al., 1975). O'Kelly and Seifert (1970) suggest haematological changes in cattle caused by B. microplus were a result of direct interference of host metabolism by secretions of toxin rather than a result of blood loss. Extended periods required for blood values of hosts to return to normal following ectoparasite eradication and the inefficiency of massive blood transfusions in restoring blood values have also been considered evidence of a toxic mechanism (review by Nelson et al., 1977).

In addition to its other possible roles, saliva may act as an anesthetic (Hudson et al., 1960; review by Beard, 1963) and as a means to aid in rapid feeding (Tatchell, 1969).

2.2 Scope of this research

Most reported experimental studies on the effects of salivary gland secretions on haemostasis are incomplete, outdated and unconfirmed or conflicting. During the past 15 years, haematologic advances have improved both understanding of haemostasis and the availability of techniques for its investigation.

This research was designed to clarify the mechanisms of action of the *Glossina morsitans* anticoagulant and to further investigate the effects of salivary gland secretions on haemostasis. There are a number of advantages to pursuing this problem. First, knowledge of the effect of salivary secretions on haemostasis *in vitro* may broaden our understanding of ectoparasite effects on host physiology *in vivo*. Second, natural inhibitors of haemostasis have provided tools to advance understanding of haemostasis and have provided compounds of potential theraputic value in man. Identification and characterization of such compounds is a necessary prerequisite to such applications. Third, the salivary glands are the reservoir of antigenic substances to which hosts respond immunologically and haematologic techniques can be used to study this aspect of host-ectoparasite interrelationships.

2.3 Materials and methods

2.3.1 General methods

Glossina morsitans morsitans (Westw.) were starved 24 to 48 hours prior to dissection. Salivary glands were removed by placing chilled flies in 0.85% NaCl and slowly pulling the head horizontally from the thorax. The glands, which remained attached to the head, were removed with forceps, without contamination from fly-gut tissue, and placed in glass vials on ice. No distinction was made between sexes, although most glands were from females. Glands were homogenized in a glass tissue grinder at a concentration of 100 pair of glands/ml of NaCl, centrifuged at 3400 g for 5 minutes at room temperature and the supernatant (SGS) removed and stored. The SGS was stored at 4°C and used within 7 days or frozen at -15°C until required. No antibacterial agents were added. This stock SGS was used for all coagulation tests unless otherwise stated. Anticoagulant activity of the supernatant varied among preparations, but for most tests, stock SGS was diluted with NaCl or CPP to obtain a conveniently measurable coagulation time prior to use. Mixtures of SGS/CPP were prepared from stock SGS immediately before study.

Citrated pooled human plasma (CPP) from 12-20 volunteers was used for most tests. Sephadex anticoagulant scans, platelet aggregation studies, platelet adherence to collagen, and euglobulin lysis times were performed using citrated plasma from me. Platelet rich and platelet poor plasma were obtained through differential centrifugation of citrated whole blood at 170 and 3000 g respectively for 10 minutes. Plastic or siliconized glassware was used for all experiments with plasma and platelets.

2.3.2 Coagulation screening tests

Blood coagulation involves a series of plasma coagulation factors, most of which circulate as proenzymes. When coagulation is initiated either through the intrinsic (surface contact) or extrinsic (tissue factor) pathways, these proenzymes are converted to enzymes. The function of each enzyme formed is to activate the next proenzyme in the sequence, resulting in a cascade effect, ultimately leading to thrombin which converts fibrinogen to insoluble fibrin (Williams et al., 1972). The purpose of these tests was to determine which coagulation factors are inhibited in the presence of the tsetse salivary gland anticoagulant. Abnormal results of coagulation tests, all of which are based on the time required for clot formation, indicate abnormalities in one or more of the clotting factors involved (Fig. 3).

2.3.2.1 Thrombin time (TT)

Principle: The thrombin time assesses the conversion of fibrinogen to fibrin following the addition of thrombin (Faulkner and King, 1970). If the thrombin time is prolonged, it may indicate a depletion or functional alteration of fibrinogen, the presence of an antithrombin, an inhibitor of fibrinogen-fibrin conversion, or an inhibitor of fibrin monomer polymerization (Fig. 3).

Method: Thrombin times were determined by the method of Fletcher et al. (1959) using 9 NIH unit bovine thrombin (Parke-Davis, Brockville, Ontario) /ml in NaCl. The normal range using a fibrometer (Baltimore Biological Laboratories) with 0.4 ml head is 10±1 seconds.

2.3.2.2 Reptilase time

Principle: Reptilase-R is a thrombin-like enzyme from the venom of

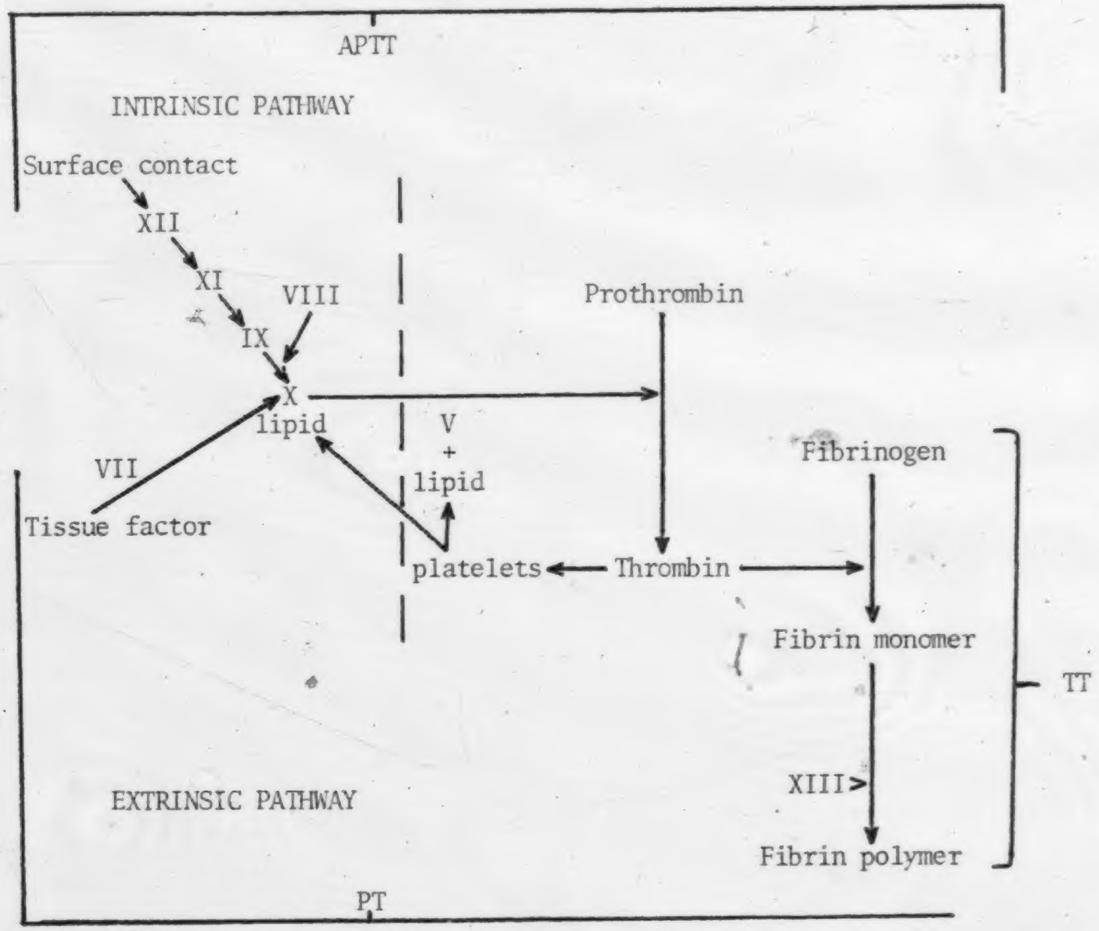

APTT
INTRINSIC PATHWAY
Surface contact
XII
XI
VIII
IX
X
lipid
V
+
lipid
Prothrombin
VII
Tissue factor
Fibrinogen
platelets
Thrombin
Fibrin monomer
TT
XIII
EXTRINSIC PATHWAY
Fibrin polymer
PT

Figure 3. Simplified coagulation scheme and screening tests for its assessment. (Lipid = platelet factor III).

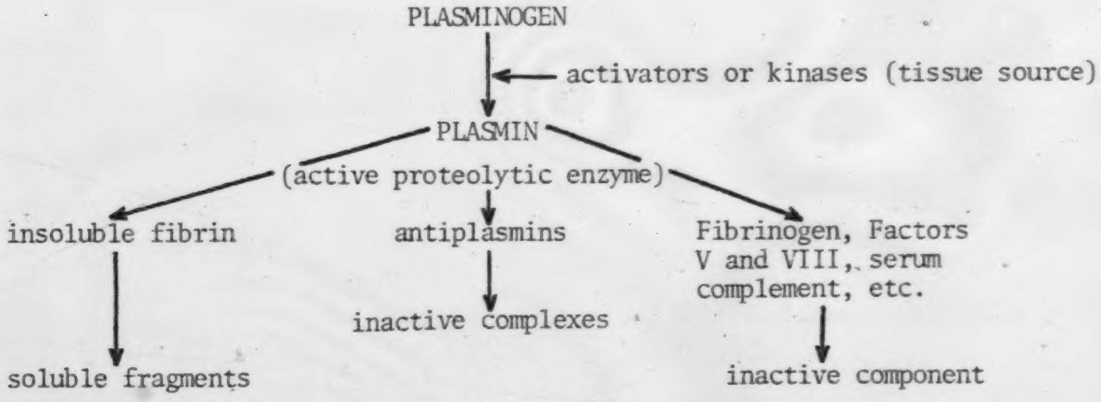

PLASMINOGEN
activators or kinases (tissue source)
PLASMIN
(active proteolytic enzyme)
insoluble fibrin
antiplasmins
Fibrinogen, Factors V and VIII, serum complement, etc.
inactive complexes
soluble fragments
inactive component

Figure 4. Human fibrinolytic enzyme system (from Williams et al., 1972).

Bothrops atrox capable of clotting fibrinogen. Unlike thrombin, which splits off fibrinopeptides A, AP, and B by its proteolytic action on fibrinogen, Reptilase releases fibrinopeptides A and AP only (Abbott Laboratory brochure, Diagnostics Division, Chicago). The effects of a test substance on the thrombin and reptilase times together, aid in determining its possible mode of action.

Method: The method outlined by Abbott Laboratory's brochure (Diagnostics Division, Chicago) was employed using a fibrometer with 0.4 ml head and substituting SGS/CPP and NaCl/CPP for CPP. Citrated normal plasma (CNP) has a normal clotting range of 18-22 seconds.

2.3.2.3 The one-stage prothrombin time (PT)

Principle: In this test, defects in the extrinsic blood coagulation pathway are measured with a mixture of plasma, tissue thromboplastin, and calcium (Williams et al., 1972). A prolongation of the PT would normally indicate a depletion, alteration, or inhibition of one or more of clotting factors V, VII, X, prothrombin, thrombin and fibrinogen (Fig. 3).

Method: The method of Cartwright (1963) was employed using commercial thromboplastin reagent (Dade Division, Florida) and a fibrometer with 0.3 ml head. The normal range is 9.9 to 11.9 seconds.

2.3.2.4 Kaolin-activated partial thromboplastin time (APTT)

Principle: This test detects deficiencies in all stages of the intrinsic clotting pathway except for platelet factor III (Fig. 3). Kaolin is used to activate factor XII, and cephalin as a platelet substitute (Biggs, 1972; Williams et al., 1972). Following incubation of kaolin and cephalin with plasma, $CaCl_2$ is added and the clotting time recorded. Prolongation of the APTT indicates a depletion, alteration, or inhibition of one or more of clotting factors XII, XI, IX, VIII, X, V, prothrombin, thrombin, and fibrinogen (Fig. 3).

Method: Tests were performed as described by Proctor and Rapaport (1961) using a chloroform extract of human brain as platelet substitute (Bell and Alton, 1954). The normal range is 35 to 48 seconds.

2.3.2.5 One-stage factor assays

Principle: Utilizing an appropriate coagulation test such as the PT or APTT, a standard activity curve is produced by varying proportions of CPP/buffer mixtures and plasma deficient in the factor being measured. These standard curves produce a straight line when log percent activity is plotted against log clotting time. Test plasma/buffer dilutions are mixed with the same proportions of deficient plasma used for the standard curve. Plotting these values on the same graph normally results in a straight line parallel to the standard curve. The percent activity of test plasma can then be read directly from the graph (Biggs, 1972). The percent activity of clotting factors following incubation with SGS will indicate whether SGS has a specific anti-clotting factor activity.

Method: With the exception of the factor II, assays were performed with naturally deficient human plasma (Warner Lambert, N.J.). Factor II assay was performed using a mixture of decalcified beef plasma and aged decalcified human serum as substrate. Factor II, V, and VII assays were performed using a modified one-stage PT procedure (Denson, 1961; Biggs and MacFarlane, 1962). Factor X assay was performed using Russell's viper venom/cephalin (Warner Lambert) and factor X free substrate plasma (Diagen Reagents, England). Factor VIII, IX, XI, and XII assays were performed using a modified APTT (Schiffman et al., 1963).

2.3.3 Tests for heparin-like and Antithrombin III activity

Heparin is an antithrombin which requires the plasma co-factor Antithrombin III for optimal anticoagulant effect (Williams et al., 1972; Rosenberg, 1975). Heparin's high negative charge and anticoagulant properties are neutralized with cationic agents such as toluidine blue and protamine sulphate (Seegers, 1967). Tests were carried out to determine if SGS has similar properties.

2.3.3.1 Protamine sulphate and toluidine blue titration

Method: The method of Dacie and Lewis (1968) was performed using protamine sulphate (Eli Lilly and Co.) and toluidine blue O (Fisher Chemical Co.) at concentrations from 50 to 1.56 mg/100 µl.

2.3.3.2 Antithrombin III requirements

Principle: As heparin requires Antithrombin III (AtIII) for optimal anticoagulant activity, tests were performed to determine if SGS has a similar requirement.

Method: The AtIII in CPP was neutralized by titration with anti-Antithrombin III sera (anti-α_2 antithrombin, #10-9047; Behring Diagnostics, Germany). Residual activity was checked by radial-immunodiffusion (*vide infra*). Antithrombin III requirements were then tested by adding sodium heparin (Allen and Hansbury, Toronto), SGS, and NaCl to equal parts of CPP/AtIII deficient plasma and recording the thrombin times on each mixture.

2.3.3.3 Total Antithrombin III determination by radial-immunodiffusion

Principle: Among its other effects on coagulation (Williams et al., 1972; Rosenberg, 1975) AtIII is a naturally occuring thrombin neutralizing protein in the blood. Its activity is a function of concentration. The amount of AtIII in plasma can be measured by diffusing test material into agarose medium containing antibody to human Antithrombin III (Fagerhol and Abildgaard, 1970). Quantitative measurement of AtIII is obtained from diameters of precipitated antibody-antigen complexes and compared with suitable standards. Glossina SGS and SGS/CPP were diffused into agar containing anti-Antithrombin III sera to determine if SGS, like AtIII, combines with anti-Antithrombin III sera, and to determine if the presence of SGS inhibits Ab-Ag precipitation zones produced by AtIII in CPP.

Method: Using a modified method of Mancini et al. (1965), anti-Antithrombin III serum was added to final concentration of 14% in agarose (Sea-Kem) in 0.1 M phosphate buffer, pH 6.5 at 55°C and poured immediately onto glass plates. Quantities of 5 µl SGS, SGS/CPP (1:1) and NaCl controls were placed into wells in the agar plates, incubated at 37°C for 20 hours under moist conditions, washed with NaCl, distilled H_2O, stained with amido black for 5 minutes, destained in methanol: acetic acid: H_2O (50:1:50) and Ab-Ag precipitation zones measured at right angles with a Bauch and Lomb measuring magnifier. The average diameters were recorded.

2.3.4 Chemical properties of SGS

2.3.4.1 Sephadex fractionation

Preswollen Sephadex G-200 (Pharmacia) was poured in a glass column of 0.35 mm inside diameter to a height of 115.5 cm. The column was eluted at 5°C with 0.15 M NaCl in 0.05 M tris (tris(hydroxy methyl) aminomethane) buffer at pH 7.5. Void and total volumes were 14.0 and 44.5 ml respectively. Fraction volumes of 0.47 ml/tube (10 drops/tube) were collected under moderately slow flow rate. Absorbance at 280 nm was obtained by adding 0.5 ml of buffer to each tube and reading on a Beckman DU-2 spectrophotometer. Blue dextran 2000, ovalbumin (Pharmacia), bovine serum albumin (Nutritional Biochem.), chymotrypsinogen A, and ribonuclease A (Sigma) were used to calibrate the column. When an anticoagulant scan was performed, every second tube was read at 280 nm while every other tube, without dilution, was used in coagulation tests. Sephadex G-75 fractionation was performed using the same size column. Bovine trypsin inhibitor, chymotrypsinogen A, cytochrome-c (Sigma) and bovine serum albumin (Nutritional Biochem.) were used to calibrate the column.

2.3.4.2 Inhibition of thrombin hydrolysis of TAME

Principle: In addition to proteolytic action on fibrinogen, thrombin has esterolytic action on synthetic substrates, particularly TAME (Sherry and Troll, 1954). This indicates that, like trypsin, thrombin cleaves the carboxyl side of basic amino acids. Tests were performed to determine if SGS inhibits thrombin's esterolytic site.

Method: Using a modified method of Sherry and Troll (1954), 0.1 ml of 40 NIH unit/ml bovine thrombin in 0.05 M tris at pH 8.95 was added to a mixture containing 50 µl SGS (100 or 200 pair of glands/ml) or NaCl and 0.9 ml of 10 mM TAME (Nutritional Biochem) in the same buffer. Absorbance at 244 nm was recorded at 30°C on a spectrophotometer. Sephadex G-75 fractions of SGS were scanned for the inhibitor and results recorded as slopes (ΔA_{244}/minute).

2.3.4.3 Incubated osmotic fragility test

Principle: Fragility of erythrocytes depends on the tonicity of the medium in which they are placed. In this test, a suspension of sterile defibrinated whole blood or washed erythrocytes in saline is incubated for 24 hours at 37°C and the resulting cells subjected to a series of saline solutions, ranging from hypertonic to hypotonic. The amount of haemolysis is determined by spectrophotometry (Brown, 1976). Salivary gland solution was incubated with erythrocytes as a means of measuring haemolytic activity of the saliva.

Method: Following the method of Brown (1976), 2.5 ml of defibrinated whole rabbit blood was incubated with 0.5 ml of SGS or NaCl for 24 hours at 37°C in sterile screw capped vials. *G. morsitans* midgut solution is known to contain digestive enzymes capable of digesting blood (Gooding, pers. commun.; reviews by Gooding, 1972a, 1975). To test the feasibility of measuring haemolytic activity with this test, 2.0 ml of sterile defibrinated blood was incubated with 0.5 ml of *G. morsitans* posterior midgut solution (67 posterior midguts/ml NaCl) from flies last fed 24 to 48 hours prior to dissection, or an equivalent amount of NaCl as control. To reduce the possibility of bacterial contamination, the midgut solution was filtered through a 0.2 µ pore size syringe filter prior to incubation. A mixture of an equivalent amount of midgut solution in saline provided a blank for the colour of the midgut solution. Absorbance at 550 nm was recorded using a Beckman DU-2 spectrophotometer.

2.3.5 Tests for fibrinolytic activity

In normal blood, a dynamic equilibrium exists between clot formation (coagulation) and destruction (fibrinolysis). The most important component of the fibrinolytic system is the blood circulating proenzyme, plasminogen, which is converted to the enzyme, plasmin, by the action of activators or kinases released from tissue sources. Plasmin functions enzymatically to digest fibrin into a number of soluble fragments (Fig. 4), but at the same time is regulated by plasma inhibitors. These experiments were performed to determine if SGS contains a fibrinolysin, a plasminogen activator, or potentiates the fibrinolytic activity of the euglobulin fraction of plasma or plasminogen.

2.3.5.1 Fibrin plates

Principle: Kabi fibrinogen (A. B. Kabi, Sweden) contains plasminogen and fibrinogen. Addition of a fibrinolysin or a plasminogen activator to agar containing clotted Kabi fibrinogen will result in an area of lysis proportional to the fibrinolytic activity. Commercially prepared Hyland plates are similar, but their clot is free of factor XIII and plasminogen. These will therefore detect the presence of a fibrinolysin only. Streptokinase is used as a standard.

Method: The method of Bishop et al. (1970) was performed using 250 mg% human fibrinogen (Grade L, A. B. Kabi, Sweden). Hyland plates were used as described for the detection of a fibrinolysin (Hyland Division, Travenol Lab., Calif.). Plasminogen was used at 1.5 u/ml (A. B. Kabi). All solutions, including Streptokinase (Behring Diagnostics, Germany) standards (5 u/ml), were added in 5 and 10 μl quantities to wells of Hyland or Kabi plates. Salivary gland solution and NaCl controls were added to plasma euglobulin fractions or plasminogen in a ratio of 1:1 or 1:2.

2.3.5.2 Euglobulin lysis time

Principle: The euglobulin fraction of plasma is relatively free of fibrinolytic inhibitors. Therefore, lysis of the clot formed from the fibrinogen in the plasma euglobulin fraction occurs relatively rapidly. An abnormally short lysis time indicates the presence of plasminogen activator activity (Williams et al., 1972).

Method: The method of Blix (1961) was performed by adding 0.1 ml of various SGS solutions or an equivalent amount of NaCl as control to plasma euglobulin fractions. The euglobulin mixtures were clotted with 50 NIH unit/ml bovine thrombin or Reptilase. Normal euglobulin lysis times are greater than 120 minutes.

2.3.6 Tests for abnormality of platelet function

Platelets are essential for normal haemostasis. They play a major role in cessation of haemorrhage from injured blood vessels and are necessary for optimal functioning of the intrinsic coagulation system. When blood vessels are damaged, platelets initially respond by adhering to the exposed subendothelial surfaces such as collagen. In doing so, they release endogenous ADP which stimulates circulating platelets to aggregate with one another as well as to those platelets already attached to the damaged endothelium. This chain of events occurs until a hemostatic plug forms, preventing further haemorrhage (Williams et al., 1972).

2.3.6.1 Platelet adherence to collagen

Principle: In the presence of EDTA, platelets will adhere to collagen but will not aggregate (review: Mustard and Packham, 1970). Platelet counts pre- and post-exposure to collagen will detect a change in adherence of platelets.

Method: Tests were performed using a newly developed technique (Mant, in press). 'Acid soluble' collagen was prepared by method of Cazenave et al. (1973a). Whole blood is drawn into 1/10 volume 2% Na_2EDTA in 0.53% saline. Platelet-rich plasma (platelet count 300,000/μl) was mixed with SGS or NaCl (7:1) and incubated at $37^{\circ}C$ for 5 minutes while being stirred at 900 rpm in a dual channel aggregation module (Payton Assoc., Scarborough, Ont.). To 0.8 ml of this mixture was added approximately 0.05 mg acid soluble collagen diluted to 0.2 ml in saline. The mixture was stirred for 10 minutes at 37°C and post-platelet counts performed (Bull et al., 1965).

$$\% \text{ adherence} = \frac{(\text{Pre count} \times 0.8) - (\text{Post count} \times 1.0)}{(\text{Pre count} \times 0.8)} \times 100$$

2.3.6.2 Platelet aggregation

Principle: Platelet aggregation may be measured *in vitro* by a reduction in absorbance of platelets in platelet-rich plasma (Williams et al., 1972). Platelet aggregants are believed to react with specific receptor sites on the platelet membrane (review: Weiss, 1975a). Primary phase aggregation involves direct aggregation of platelets by the aggregating agent. Most agents also induce release of endogenous platelet ADP which mediates second phase aggregation (collagen aggregates platelets only via released ADP).

Method: Platelet count (Bull et al., 1965) and platelet aggregation (Born, 1962; O'Brien, 1962) were performed using standard techniques. Aggregating agents used were acid soluble collagen (Cazenave et al., 1973a: Sigma bovine achilles tendon; 1:25, 1:50, 1:100, 1:200, 1:300, dilution of the stock 'solution'), ADP (Sigma: 100, 10, 2 μM concentrations), adrenalin (Eli Lilly and Co., Copenhagen; 500, 50, 25, 10 and 1 μM concentrations), bovine thrombin (Parke-Davis; 10, 3.5, 2.5 NIH unit/ml), and ristocetin (Lundbeck and Co., Copenhagen; 20, 25, 12 mg/ml). All dilutions were in saline with final concentrations in plasma, 1/10 of those indicated. Aggregating agents were added in 50 μl quantities to 0.45 ml of prewarmed SGS/PRP or NaCl/PRP at 37°C. Incubation time of SGS with PRP did not affect platelet aggregation.

Recorder levels of 0 and 100% transmittance were set with PRP and PPP respectively. Platelet aggregations were performed on 7 different occasions using different SGS solutions each time. Analysis of aggregation curves was performed using 3 different measurements (Zucker et al., 1972):

1. Reaction time: represents the delay in seconds between the addition of aggregating agents and the initial increase in light transmission through platelet-rich plasma.
2. Slope: expressed as percent increase in light transmission per minute, measured at the maximal rate of change.
3. Maximum: maximum increase in light transmission following addition of aggregating agent (regardless of platelet deaggregation) expressed as a percent of 100% transmittance limits set with PPP.

Example of measurements used in analysis is shown in Fig. 5. Triplicate aggregations were performed at each concentration of each aggregating agent. Results were analyzed by ANOVA (program ANOFPR, University of Alberta Computing Services) and graphing mean values.

2.3.6.3 Scanning electron microscopy (SEM) of platelets

Principle: Aggregating agents induce a platelet shape change from smooth discs to spheres with pseudopodia (Biggs, 1972). These pseudopodial extensions increase the effective diameter of the platelet, increase the possibility of platelet-platelet contact, and ultimately lead to platelet aggregation (Bangham, 1964). In order to correlate platelet surface structure with aggregation response, platelets were examined by SEM in the presence of SGS.

Method: The method of Walsh and Barnhart (1973) was performed as

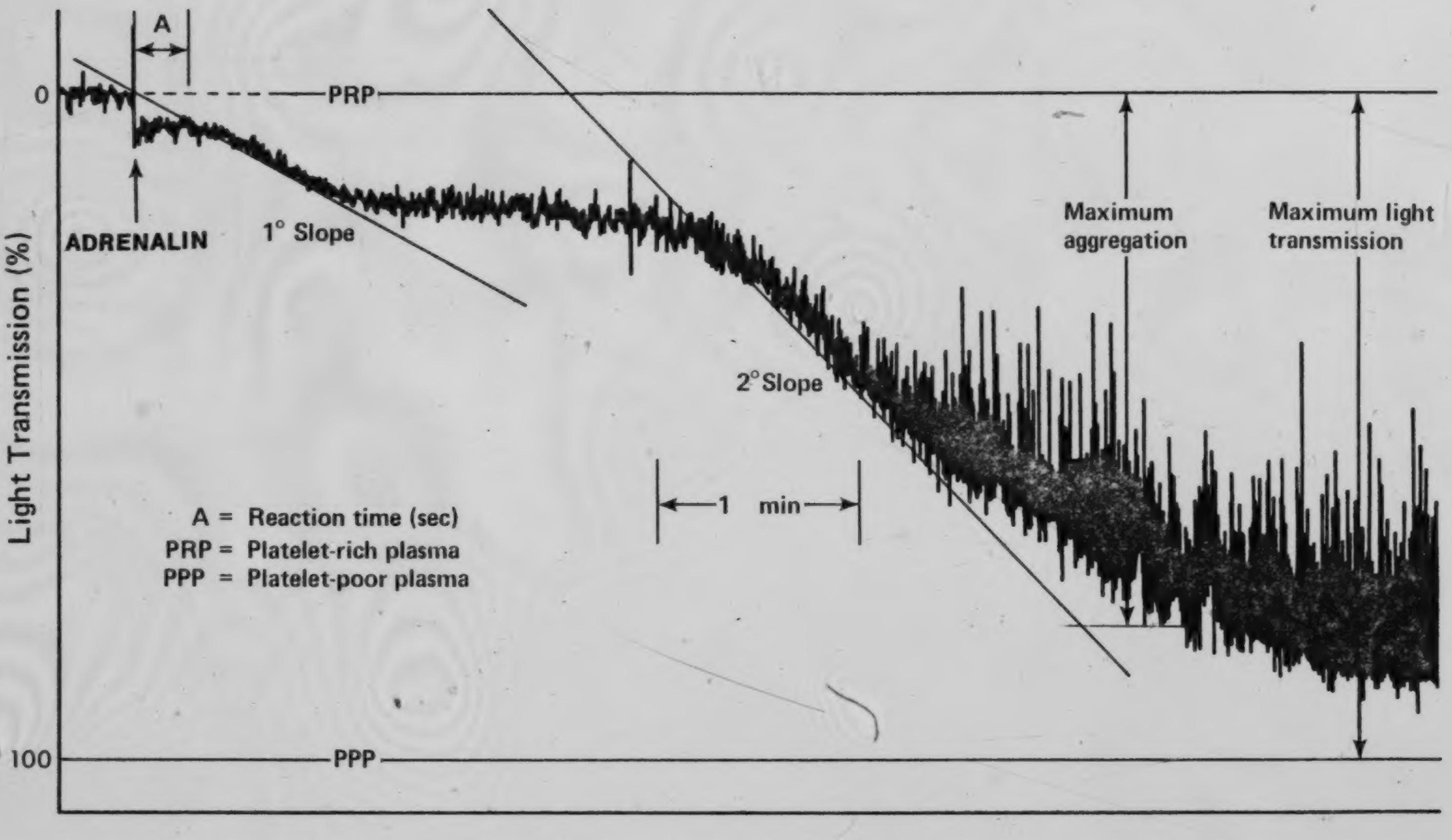


Figure 5. Example of measurements used in analysis of platelet aggregation curves (from Zucker et al., 1972).

described for ADP, with the exception that dextrose was not present. Samples were prepared in duplicate on only one occasion. Samples of SGS/PRP and NaCl/PRP, stirred for the same length of time at 37°C as samples with aggregating agents, served as controls. Final concentrations of platelet aggregants were: 1 μM ADP, 5 μM adrenaline, 0.35 NIH unit/ml thrombin, and 1:500 acid collagen. Following aggregation, plasma mixtures were fixed immediately in glutaraldehyde, placed on glass cover slips with or without formvar coating, covered with a thin film of gold-palladium and viewed at 25 Kv with a Cambridge S-4 stereoscan electron microscope.

2.3.7 Neutralization of anticoagulant with rabbit antisera

Two serum samples from each of 2 Flemish Giant X French Lop-eared rabbits were removed from storage at -15°C. One serum from each rabbit was taken prior to the time rabbits were exposed to G. morsitans. The other serum sample from each rabbit was obtained after heavy tsetse exposure and contained naturally produced antibodies to SGS (see section 3.4.2). All 4 sera were heated at 56°C for 30 minutes and centrifuged for 30 minutes at 15,000 g. Rabbit sera (0.15 ml) was added to SGS/CPP or NaCl/CPP (0.85 ml) and incubated at 37°C for 40 minutes. A third portion of CPP served as control. Prothrombin (PT), TT, and APTT were performed on sera/plasma mixtures.

2.4 Results

2.4.1 Anticoagulant specificity

2.4.1.1 Coagulation screening tests

Glossina morsitans SGS prolonged the PT, TT, and APTT (Fig. 6). Dilution of SGS in saline reduced the prolongation of the PT and TT, and to a lesser extent the APTT. The reptilase time was not prolonged in the presence of SGS (Table 1). The normal reptilase time in the presence of a prolonged thrombin time indicates the anticoagulant of SGS acts as an antithrombin rather than an inhibitor of fibrinogen, fibrinogen/fibrin conversion, or fibrin monomer polymerization (Latallo and Teisseyre, 1971).

2.4.1.2 One-stage factor assays

Results of all factor assays were similar (Table 2). As SGS was diluted in buffer, the measureable factor activity approached 100%. Each clotting factor assay gave lower results in the presence of more concentrated SGS, the pattern being similar for each clotting factor. With the one-stage assays used, this phenomenon can be explained by anticoagulant activity affecting the test system, but not specifically directed against the clotting factor being measured (Margolis and Bruce, from Williams et al., 1975).

2.4.1.3 Comparison with heparin

Unlike heparin, the anticoagulant activity of SGS was not neutralized by protamine sulphate or toluidine blue (Table 3), and does not require the plasma co-factor, antithrombin III for prolongation of the clotting time (Table 4). Slight prolongation of the TT by heparin in CPP/AtIII deficient (Table 4) may be the result of residual AtIII activity, since specific antisera against AtIII neutralizes only 90% of

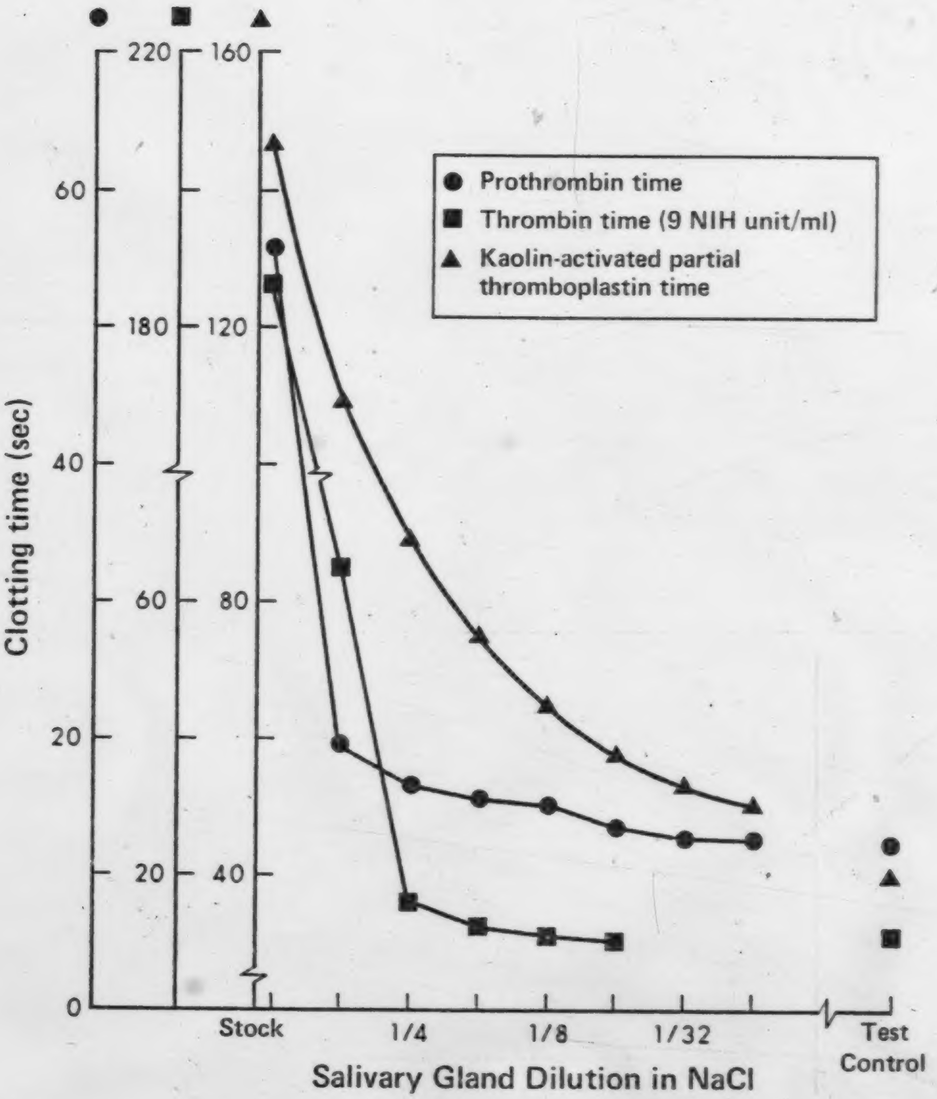


Figure 6. Effect of *G. morsitans* salivary anticoagulant on the prothrombin, thrombin, and kaolin-activated partial thromboplastin times.

Table 1. Reptilase-R clotting time in the presence of SGS.

	test mixture (ml)				clotting time[a] (seconds)
	NaCl	SGS	CPP	Reptilase	
Test check	---	---	0.3	0.1	17.9
Control	0.05	---	0.3	0.1	16.9
Experimental	---	0.05	0.3	0.1	15.5

[a] Clotting times are averages of 4 replicates.

Normal range for CNP is 18 - 22 seconds.

Table 2. One-stage factor assay results in the presence of SGS.

Factor assay	Percent factor activity of SGS/buffer dilutions					
	1/5	1/10	1/20	1/40	1/80	1/100
II	---	68	78	88	94	---
V	---	---	---	94	96	100
VII	63	90	92	---	---	---
VIII	---	46	54	---	---	95
IX	---	30	40	---	---	90
X	---	66	87	100	---	---
XI	---	22	28	---	---	90
XII	---	64	76	---	---	98

Table 3. Effect of protamine sulphate and toluidine blue on anticoagulant activity of SGS.

protamine sulphate[a] or toluidine blue conc'n	Thrombin time (3 NIH unit/ml) (Sec)[b]			
	Protamine sulphate		Toluidine blue	
mg%	NaCl/CPP	SGS/CPP	NaCl/CPP	SGS/CPP
4.55	19.3	57.3	21.4	34.2
2.27	---	55.6	---	33.3
1.14	---	50.8	---	33.2
0.57	---	47.0	---	32.4
0.28	---	43.5	---	32.1
0.14	15.3	42.8	22.2	37.7
0.0	17.9	43.6	21.7	31.2

[a] Final conc'n in plasma. Incubated with plasma for 30 seconds.

[b] Clotting times are means of duplicates.

Table 4. Effect of Antithrombin III deficient CPP on the anticoagulant activity of SGS.

Anticoagulant[a]	Thrombin time (9 NIH unit/ml) (seconds)	
	Normal CPP	CPP/AtIII deficient
No addition	10.5	11.3
NaCl	10.0	12.5
Heparin	> 360	15.5
SGS	209.4	> 360

[a] Heparin, SGS and NaCl were added to plasma in a ratio of 1:2 respectively.

its activity (Rosenberg, 1975). Prolongation of the TT in the presence of SGS/CPP/AtIII deficient may be the result of diluting plasma clotting factors by addition of SGS (Table 4). The prolonged TT of NaCl/CPP and SGS/CPP at higher concentrations of protamine sulphate (Table 3) is probably due to the anticoagulant action which occurs at high concentrations (Seegers, 1967).

2.4.1.4 Radial-immunodiffusion in agar containing anti-AtIII sera

Results of radial-immunodiffusion of SGS into agar containing human anti-Antithrombin III serum indicate SGS does not contain material antigenically similar to AtIII, nor does it contain material which inhibits the Ab-Ag reaction of AtIII with its antisera (Fig. 7).

2.4.2 Chemical properties of SGS

2.4.2.1 Incubation time of SGS with CPP

Incubation of SGS/CPP and NaCl/CPP controls at 37°C for a period of 4 hours did not result in further prolongation of clotting times from clotting times obtained initially (Table 5).

2.4.2.2 Storage stability of SGS at +4° and -20°C

Anticoagulant activity of homologous aliquots of SGS stored at +4° and -20°C did not decline during a 1 month period (Table 6). Tests at room temperature were unsuccessful due to bacterial contamination.

2.4.2.3 Sephadex fractionation of SGS

Five separate fractionations of SGS on Sephadex G-200 demonstrate 3 major protein peaks (Fig. 8). The first peak eluted in the void volume. The third peak eluted protein of low molecular weight

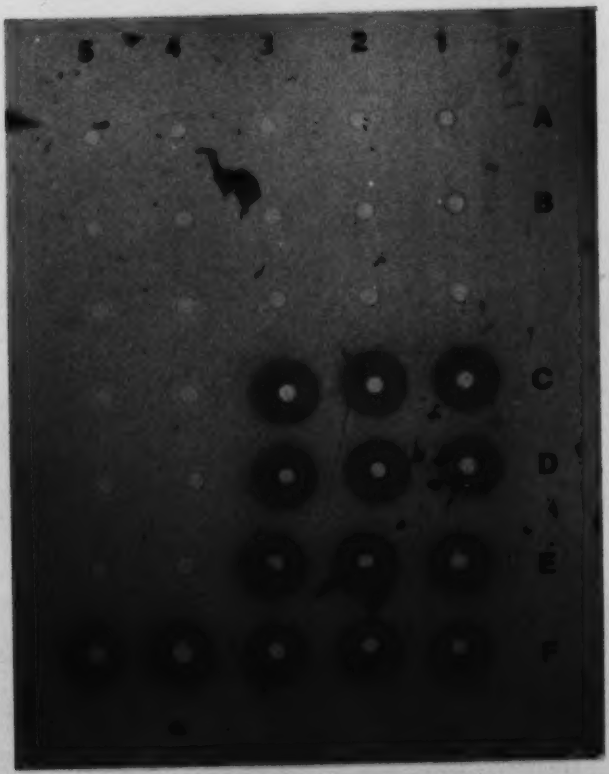


Figure 7. Radial-immunodiffusion of SGS and SGS/CPP in agarose containing anti-Antithrombin III serum.

Horizontal: No. 1 to 3 are doubling dilutions from stock to 1/4 dilution of NaCl and SGS. No. 4 and 5 are two further doubling dilutions of NaCl.

Vertical: A) NaCl B) SGS C,D,E) SGS/CPP (1:1) F) NaCl/CPP (1:1)

Table 5. Effect of incubation time on anticoagulant activity of SGS in CPP.

Coagulation[a] test	SGS dilution	Clotting time[b] (sec) with incubation (hr) at 37°C		
		0 hr	1 hr	4 hr
Prothrombin time	stock	14.8	13.8	14.3
	1/8	12.8	12.0	12.1
Kaolin-activated partial thromboplastin time	stock	90.5	85.1	95.1
	1/8	58.1	55.2	59.9
Thrombin time				
9 NIH unit/ml	stock	164.9	191.8	177.8
	1/8	12.8	10.2	10.0
3 NIH unit/ml	stock	>500	>500	>500
	1/8	41.0	39.0	39.6

[a] Control times: PT, 11.8 sec.; APTT, 40.5 sec.; TT 9 NIH unit/ml, 10.3 sec.; 3 NIH unit/ml, 19.5 sec.

[b] Clotting times are means of duplicates.

Table 6. Stability of SGS anticoagulant activity over 1 month at +4° and -20°C

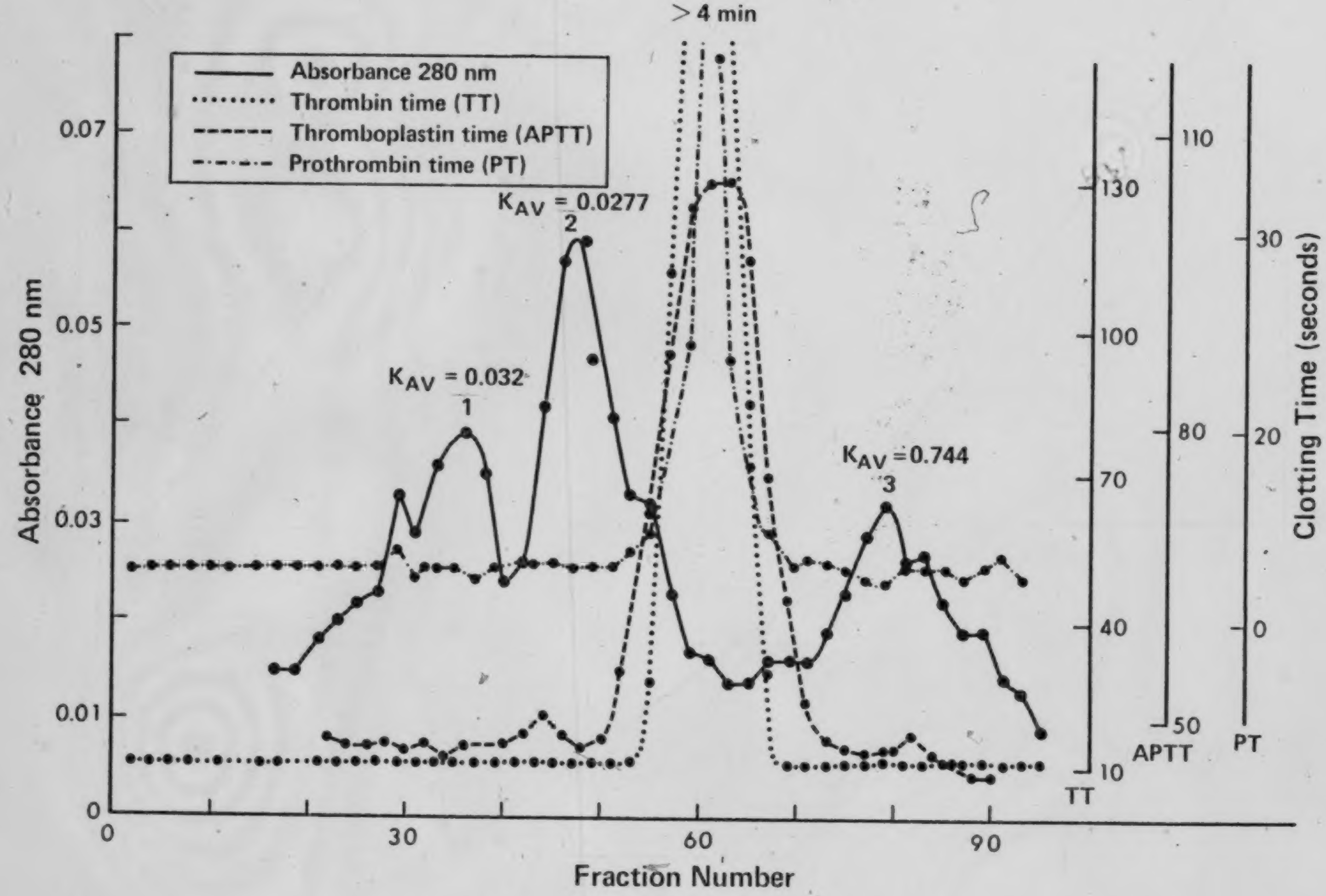
Temperature[a]	Time[b] (days)	Prolongation of clotting time[c] (sec) from control (NaCl)		
		PT	APTT	TT
+4°C	0	3.2	43.1	197.3
	1	2.2	41.8	191.2
	13	2.5	39.1	137.1
	36	2.9	50.3	174.9
-20°C	0	5.2	56.0	> 200
	1	5.0	62.2	> 200
	9	4.5	54.4	> 200
	32	3.4	60.1	> 200

[a] Different preparations of SGS were used for each temperature.

[b] NaCl/CPP and SGS/CPP were mixed from separate, although homologous aliquots of SGS and NaCl on the day of use.

[c] Means of duplicate times were subtracted.

Figure 8. Sephadex G-200 fractionation and anticoagulant scan of SGS. Three protein peaks, none of which correlate with anticoagulant activity, are shown. Anticoagulant scan using PT, TT, and APTT indicates only 1 peak of activity.


Absorbance 280 nm
Thrombin time (TT)
Thromboplastin time (APTT)
Prothrombin time (PT)
> 4 min
K_{AV} = 0.032
1
K_{AV} = 0.0277
2
K_{AV} = 0.744
3
Absorbance 280 nm
0
0.01
0.03
0.05
0.07
Fraction Number
0
30
60
90
TT
10
40
70
100
130
APTT
50
80
110
PT
0
20
30
Clotting Time (seconds)

(< 5000). A middle peak of protein with a molecular weight of 27-32000 daltons was eluted in tubes #46 - 49. One peak of anticoagulant activity, not corresponding to any of the major protein peaks (Fig. 8), eluted at a molecular weight of 11,000 to 13,000 (Fig. 9).

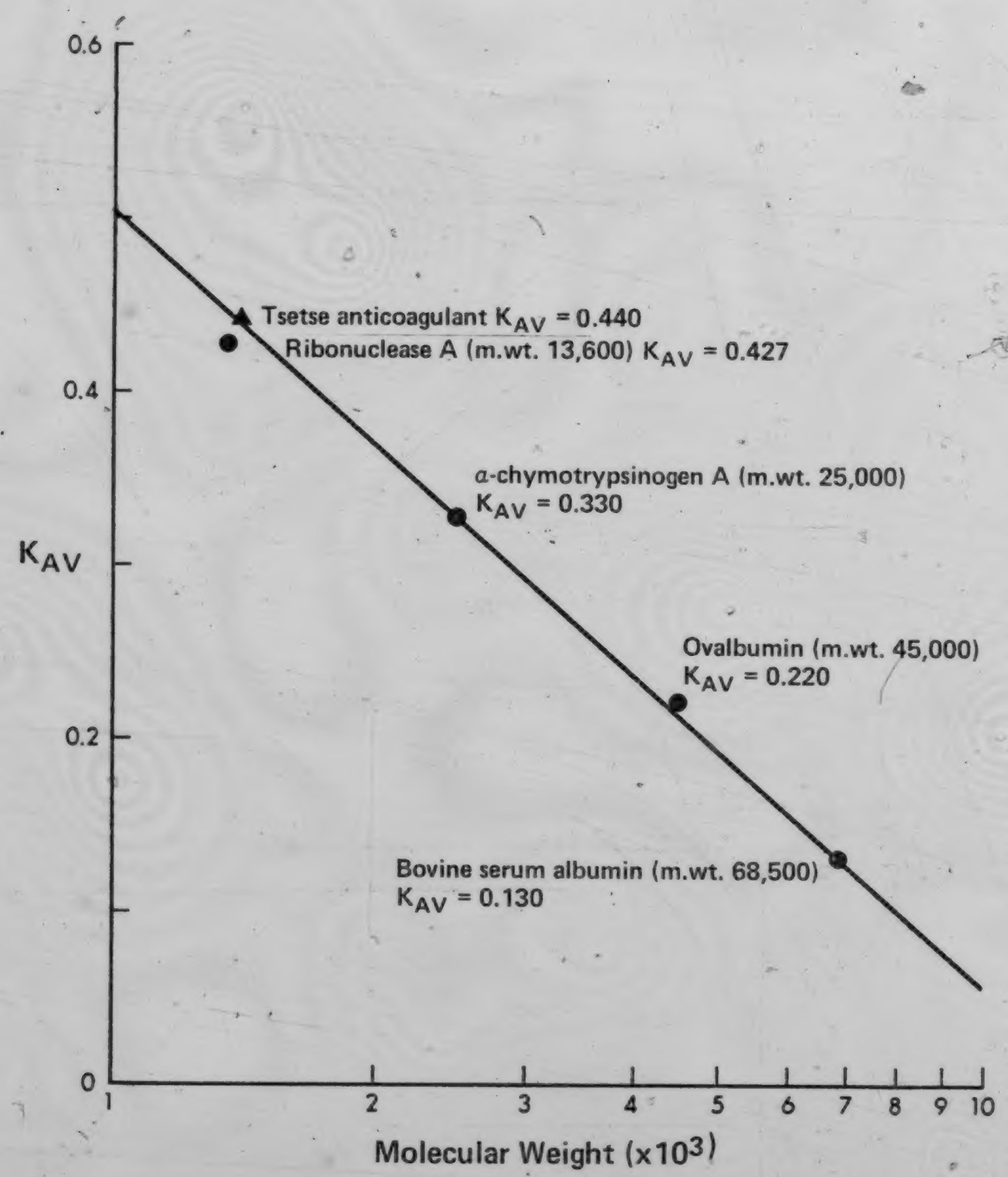


Figure 9. Standard curve of Sephadex G-200 for molecular weight determination of SGS-anticoagulant activity.

2.4.2.4 Heat stability of SGS

Clotting times of stock SGS after incubation at 98°C were inconsistent, probably due to effects of denatured proteins. To overcome this problem, Sephadex fractionation was used to partially purify the anticoagulant. Maximum anticoagulant activity appeared in fractions #54 and #61 of Sephadex G-75 (Fig. 11) and G-200 (Fig. 8) columns respectively. Coagulation times of 5 fractions from the G-75 column during 1 hour at 98°C are presented in Figure 10a and 10b. The TT of tube #54 fell after 30 minutes, but became more prolonged than the initial TT after 60 minutes (Fig. 10a). The TT of the other 4 fractions, with the exception of tube #50, did not change with prolonged heating. The APTT of the same Sephadex fractions did not change (Fig. 10b).

2.4.2.5 Inhibition of thrombin hydrolysis of TAME

G. morsitans SGS inhibited thrombin hydrolysis of TAME, with maximum inhibition occurring in the same fractions as maximum anticoagulant activity (Fig. 11). Concentrations of SGS as high as 200 pair of glands/ml did not completely inhibit hydrolysis, although at lower thrombin concentrations, inhibition was more pronounced.

2.4.2.6 Incubated osmotic fragility test for haemolysis

Incubation of whole defibrinated blood at 37°C for 24 hrs with SGS did not alter the osmotic fragility of rabbit erythrocytes (Fig. 12a). Similar results were obtained with washed rabbit erythrocytes. A homogenate of *G. morsitans* midguts incubated under the same conditions resulted in complete lysis of erythrocytes regardless of saline concentration (Fig. 12b).

2.4.3 Fibrinolytic activity of SGS

Neither fibrinolytic activity nor plasminogen activator were

Figure 10. Heat stability of Sephadex G-75 fractions containing anticoagulant activity of SGS.

10a. Thrombin times of SGS/CPP fractions

10b. Kaolin-activated partial thromboplastin times of SGS/CPP fractions

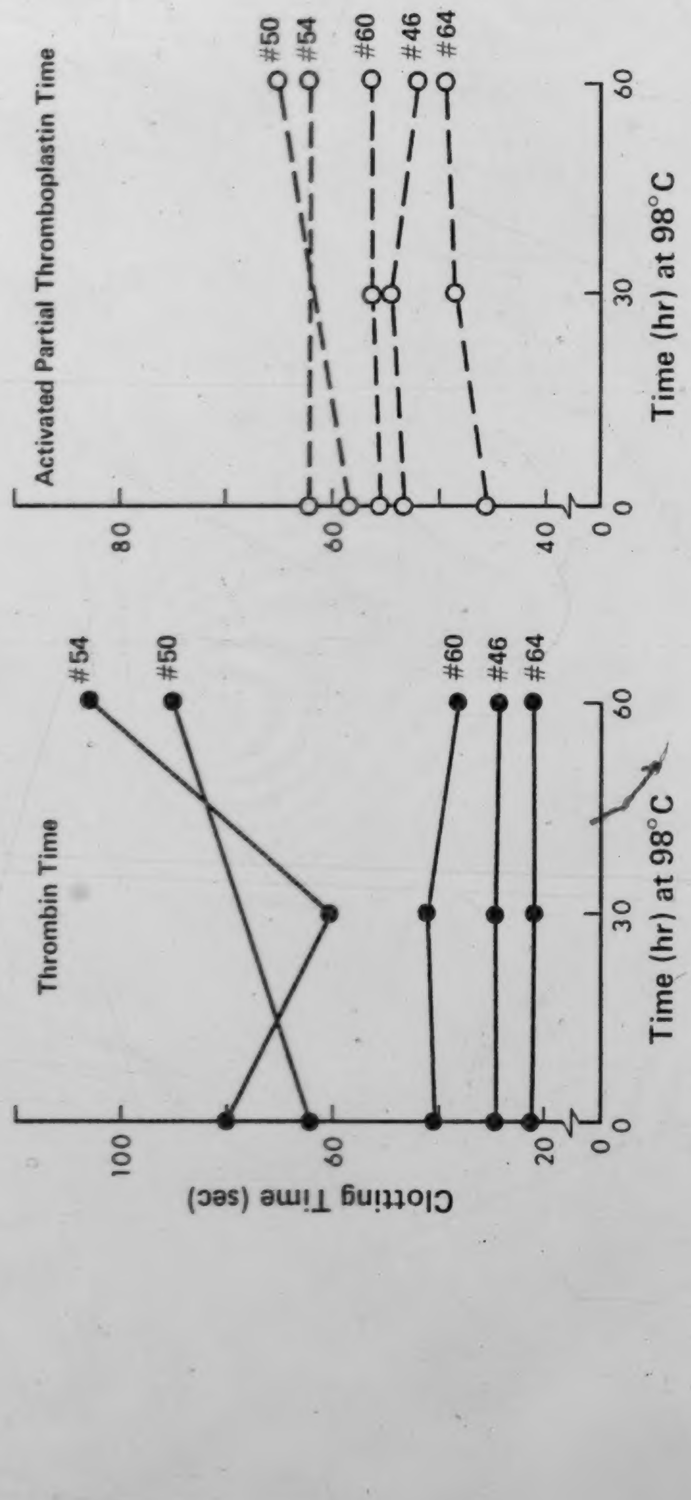
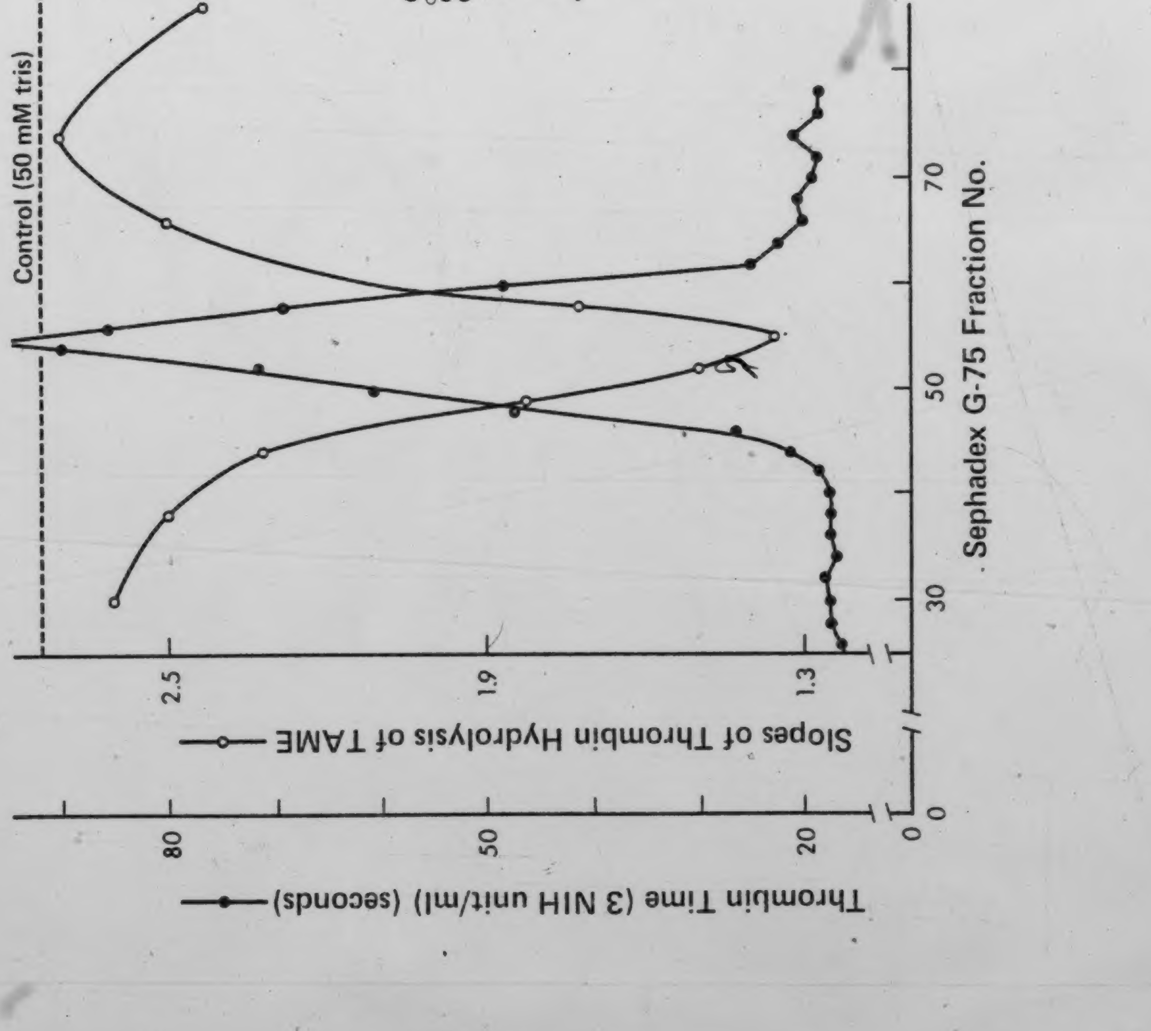

Thrombin Time
Activated Partial Thromboplastin Time
Clotting Time (sec)
100
60
20
0
80
60
40
0
Time (hr) at 98°C
Time (hr) at 98°C
0
30
60
0
30
60
#54
#50
#60
#46
#64
#50
#54
#60
#46
#64

Figure 11. Sephadex G-75 scan for inhibition of thrombin hydrolysis of TAME. Maximum anticoagulant activity and maximum inhibition of thrombin hydrolysis of TAME occurred in the same fractions. Insert: example of hydrolysis of TAME assay and slopes of hydrolysis which are plotted with anticoagulant scan.


Thrombin Time (3 NIH unit/ml) (seconds)
Slopes of Thrombin Hydrolysis of TAME
Control (50 mM tris)
80
50
20
0
2.5
1.9
1.3
0
30
50
70
Sephadex G-75 Fraction No.

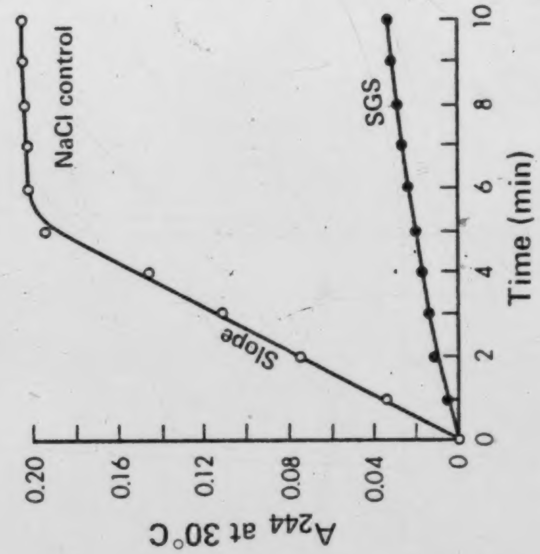
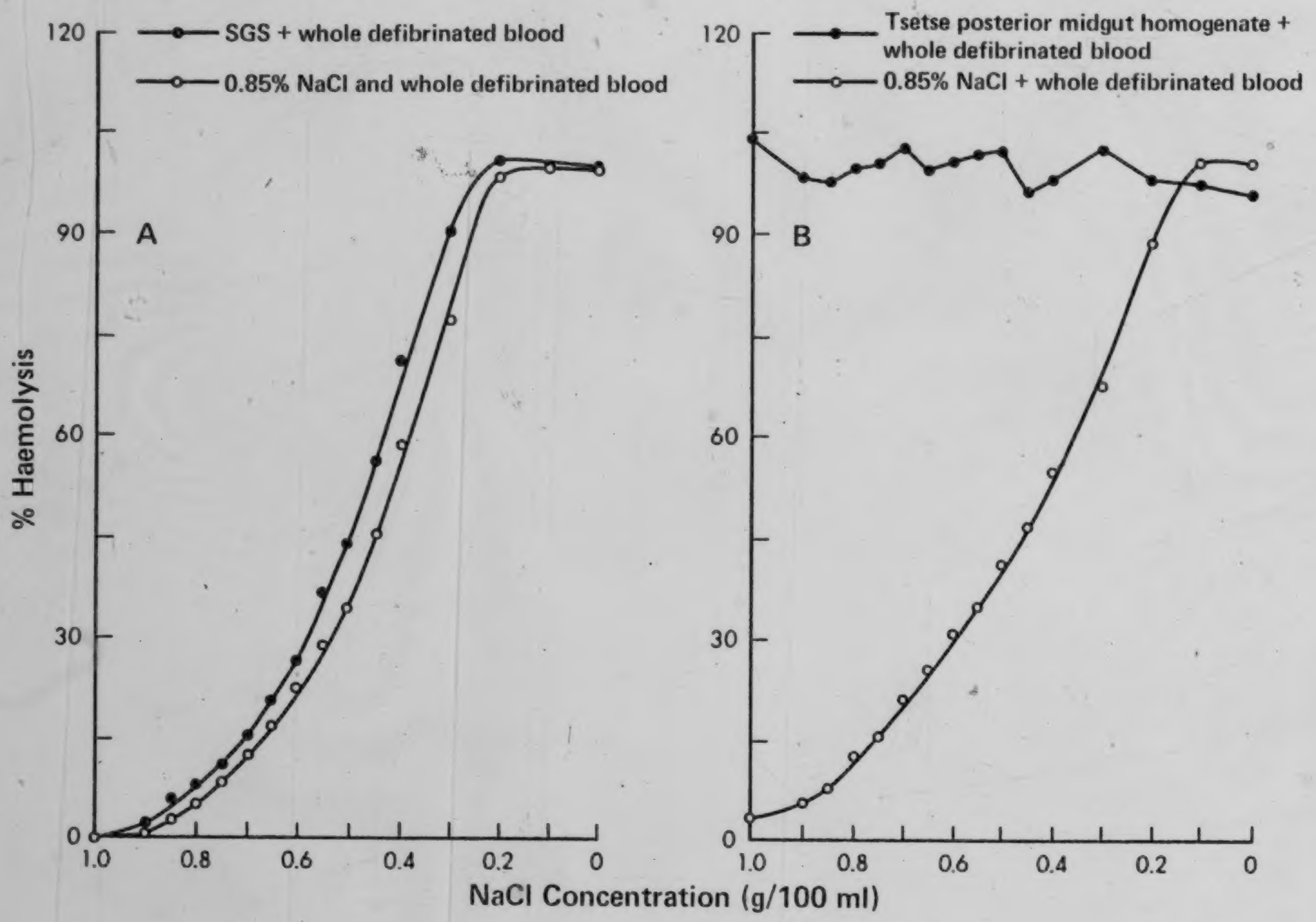

NaCl control
SGS
Slope
0.20
0.16
0.12
0.08
0.04
0
A244 at 30°C
0
2
4
6
8
10
Time (min)

Figure 12. Haemolytic activity of SGS and posterior midgut solution.

12a. SGS (180 pair of glands/ml) and NaCl controls

12b. *G. morsitans* posterior midgut solution (67 posterior midguts/ml) and NaCl controls



% Haemolysis
120
90
60
30
0
A
SGS + whole defibrinated blood
0.85% NaCl and whole defibrinated blood
1.0
0.8
0.6
0.4
0.2
0
B
Tsetse posterior midgut homogenate + whole defibrinated blood
0.85% NaCl + whole defibrinated blood
120
90
60
30
0
1.0
0.8
0.6
0.4
0.2
0
NaCl Concentration (g/100 ml)

identified when SGS was added to Kabi and Hyland fibrin plates (Table 7). No potentiation of lytic activity occurred when SGS was added to the euglobulin fraction of plasma or to plasminogen (Table 7). One gut from an unfed teneral tsetse produced a large lytic zone on a Kabi plate (Fig. 13).

Euglobulin lysis times substantiated the absence of fibrinolytic activity in SGS. In two experiments run in triplicate, euglobulin lysis times with doubling dilutions of SGS in saline, added to the plasma euglobulin fraction and clotted with thrombin or reptilase, were similar to those of saline controls (> 120 minutes).

2.4.4 Effect of SGS on platelets

2.4.4.1 Platelet adherence to collagen

Platelet adherence was measured in 4 samples of PRP, cumulating 12 replicates for both control (NaCl) and test (SGS) plasmas. Mean values (±S.D.) of percent adherence were: SGS: 41.5 ± 4.8; NaCl: 45.8 ± 6.8, and do not differ significantly (Student's t, $P > 0.05$). These results demonstrate SGS does not affect platelet adherence to collagen.

2.4.4.2 Platelet aggregation

Acid collagen-induced aggregation: Platelet aggregation in the presence of SGS was inhibited (Fig. 14a, 15). Maxima were significantly ($P < 0.05$) reduced in the presence of SGS, the inhibition being independent of acid collagen concentration (Appendix B, Table B1). Slopes were also significantly reduced in PRP containing SGS, however inhibition was dependent upon the concentration of acid collagen used (Appendix B, Table B2).

ADP-induced aggregation: In the presence of SGS, platelet aggregation

Table 7. Fibrinolytic activity of SGS on fibrin plates.

	Zone of lysis (mm^2) in agar					
	Kabi plate[a]			Hyland plate		
	no addition	euglobulin	plasminogen	no addition	euglobulin	plasminogen
No addition	----	25.4	41.0	----	12.6	30.8
0.85% NaCl[b]	0	22.9	39.3	0	10.9	25.5
SGS	0	21.0	37.8	0	10.6	25.5

[a] SGS/CPP and NaCl/CPP mixtures were not fibrinolytic

[b] Euglobulin plasma fraction and plasminogen were mixed with equivalent amounts of SGS or NaCl.

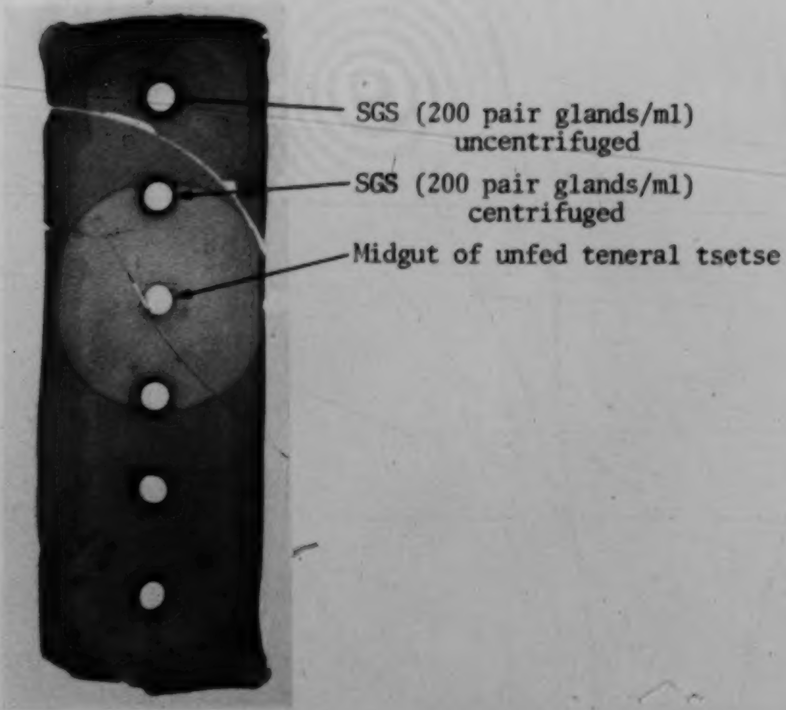

SGS (200 pair glands/ml)
uncentrifuged
SGS (200 pair glands/ml)
centrifuged
Midgut of unfed teneral tsetse

Figure 13. Lytic activity of SGS and posterior midgut solution on fibrin plates. Plates containing fibrinogen and plasminogen were incubated for 24 hours at 37°C.

Figure 14. Representative aggregation curves and the effect of SGS.

A. Representative aggregation curves induced by acid collagen at 1:500 and 1:2000 final concentration from stock solution. Both slope and maxima are inhibited.

B. Representative aggregation curves induced by ADP at 10 and 1 μM final concentration in plasma. Note deaggregation in SGS/PRP at both concentrations. The secondary wave of aggregation is abolished in PRP containing SGS.

C. Representative aggregation curves induced by adrenaline at 5 and 1 μM concentration. Note deaggregation in SGS/PRP at 5 μM.

D. Representative aggregation curves induced by ristocetin at 2.0 and 1.2 mg/ml final concentration. No statistical difference between SGS and NaCl controls.

E. Representative aggregation curves induced by thrombin at 5.0 and 0.35 NIH unit/ml final concentration. No aggregation occurred in SGS/PRP, regardless of the thrombin concentration added.

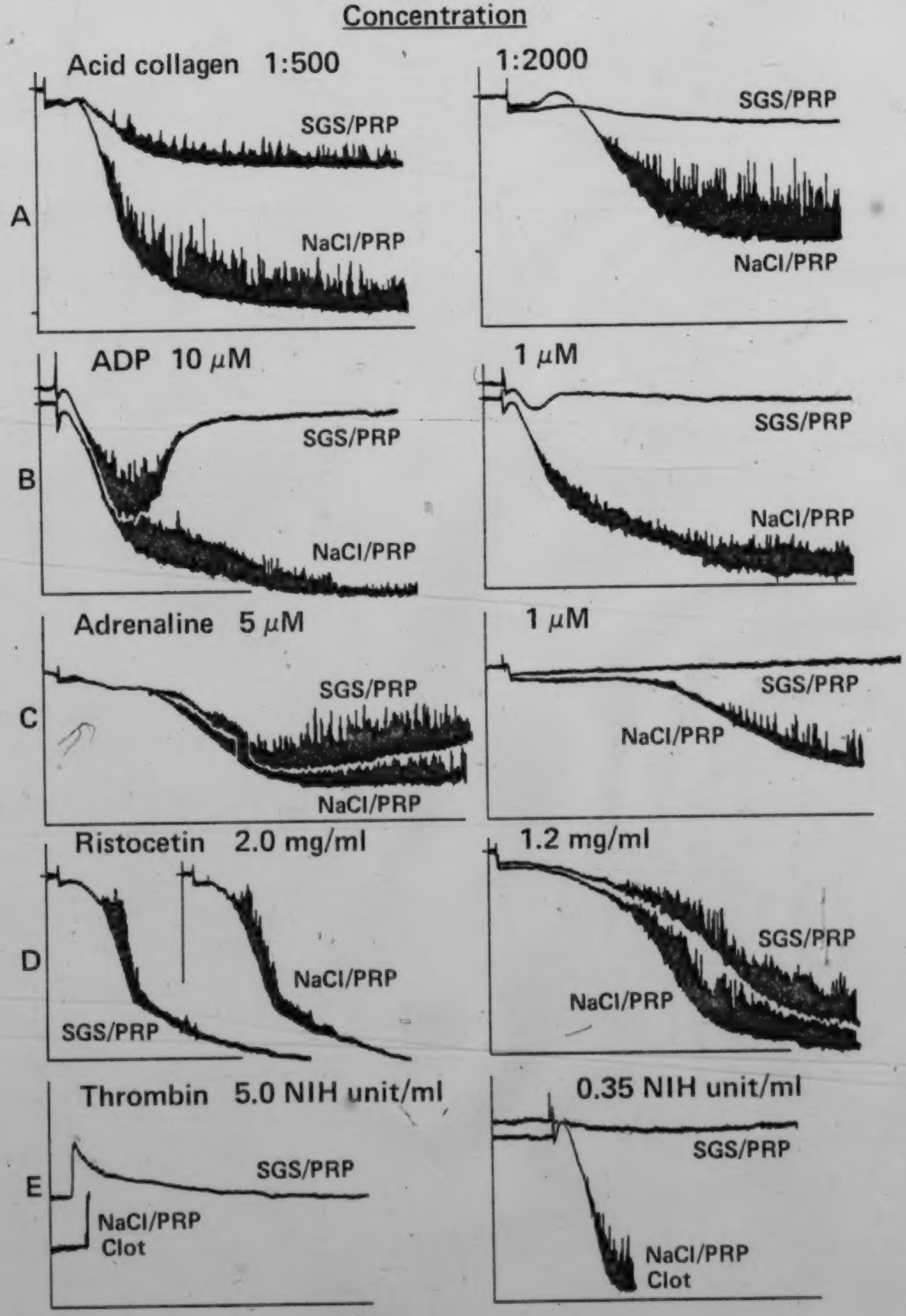
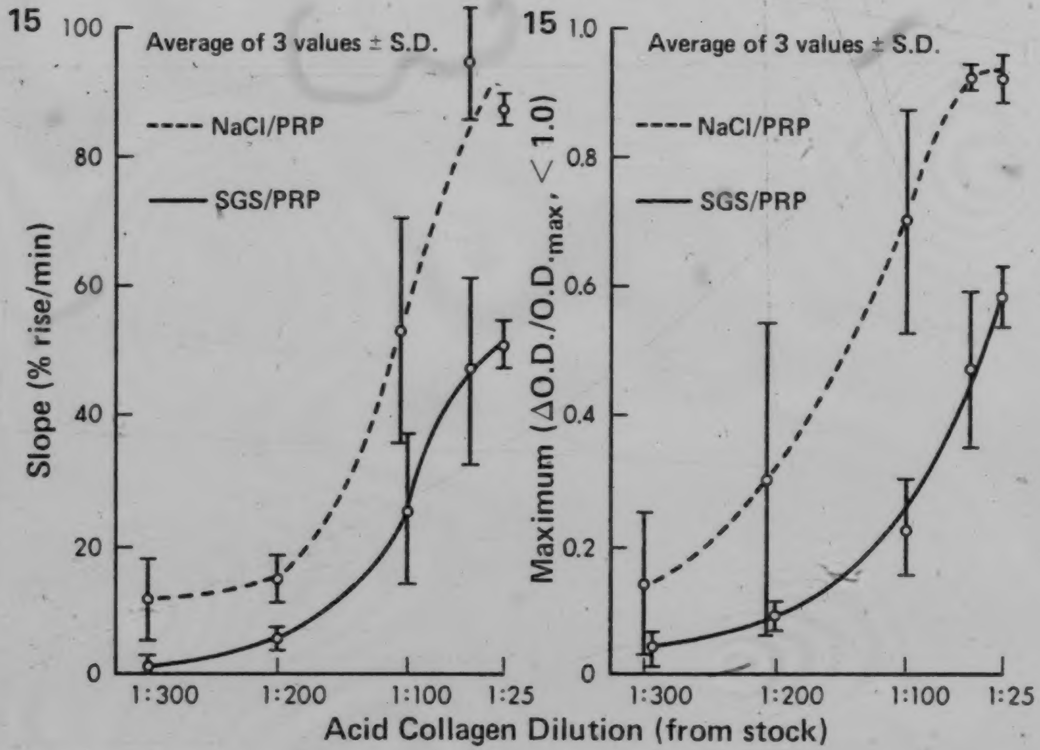

Concentration
Acid collagen 1:500
1:2000
SGS/PRP
NaCl/PRP
A
ADP 10 μM
1 μM
SGS/PRP
NaCl/PRP
B
Adrenaline 5 μM
1 μM
SGS/PRP
NaCl/PRP
C
Ristocetin 2.0 mg/ml
1.2 mg/ml
SGS/PRP
NaCl/PRP
D
Thrombin 5.0 NIH unit/ml
0.35 NIH unit/ml
SGS/PRP
NaCl/PRP Clot
E

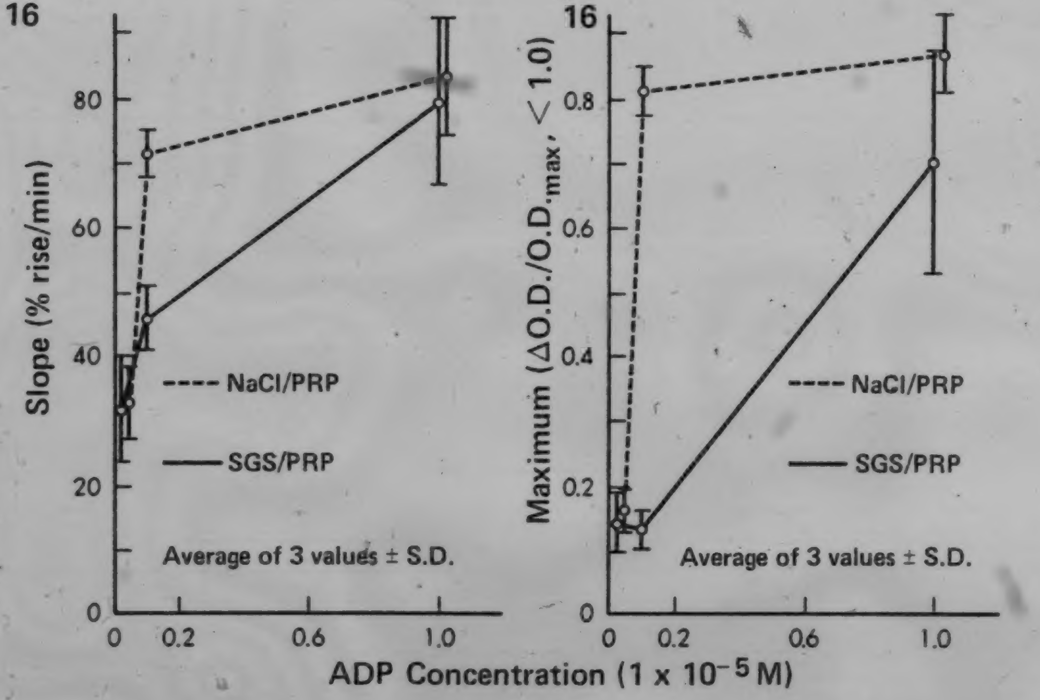
Figure 15. Slopes and maxima of aggregation curves induced by acid collagen.

Figure 16. Slopes and maxima of aggregation curves induced by ADP.

15


Average of 3 values ± S.D.
NaCl/PRP
SGS/PRP
Slope (% rise/min)
0
20
40
60
80
100
1:300
1:200
1:100
1:25
Maximum (ΔO.D./O.D.max, < 1.0)
0
0.2
0.4
0.6
0.8
1.0
Acid Collagen Dilution (from stock)

16


Slope (% rise/min)
0
20
40
60
80
NaCl/PRP
SGS/PRP
Average of 3 values ± S.D.
0
0.2
0.6
1.0
Maximum (ΔO.D./O.D.max, < 1.0)
0
0.2
0.4
0.6
0.8
ADP Concentration (1 x 10⁻⁵ M)

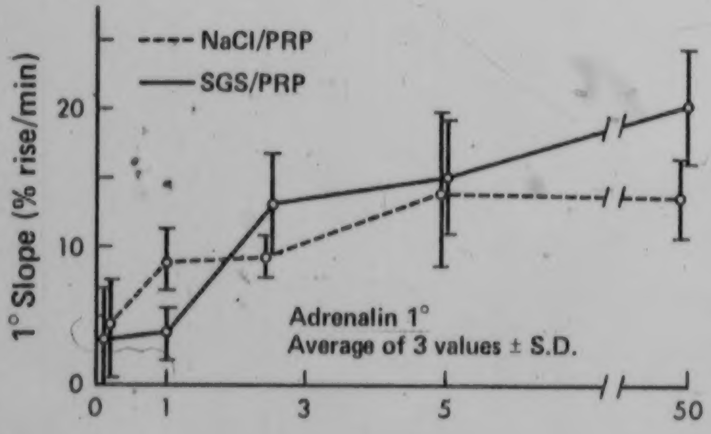
was inhibited (Fig. 14b, 16). Maximum inhibition occurred at 1 μM ADP. Critical concentrations between 1 and 2 μM ADP are normally required to induce a distinct secondary wave of aggregation (MacMillan, 1966). At 1 μM ADP, the secondary wave of PRP containing NaCl was only partially distinguishable, while the secondary wave of PRP containing SGS was abolished (Fig. 14b). Deaggregation is known to occur in human PRP at concentrations between 0.2 and 1 μM ADP (review by Weiss, 1975a). Deaggregation occurred at all concentrations (10, 1, 0.2 μM) of ADP in PRP containing SGS, but only at 0.2 μM in control plasma (Fig. 14b). Maxima of aggregation curves were significantly ($P < 0.05$) reduced in PRP containing SGS, with inhibition being dependent on ADP concentration (Appendix B, Table B3). Slopes of aggregation curves were also significantly reduced, independent of ADP concentration (Appendix B, Table B4).

Adrenalin -induced aggregation: Maxima and slopes of primary aggregation were not affected in plasma containing SGS (Fig. 14c, 17; Appendix B, Table B1, B2). Secondary slopes and maxima were inhibited, and occasionally absent at lower adrenalin concentrations, in plasma containing SGS (Fig. 14c, 18). Slopes were significantly reduced ($P < 0.05$) regardless of adrenalin concentration (Appendix B, Table B2). Maxima were also significantly reduced in plasma containing SGS, with inhibition being dependent on adrenalin concentration (Appendix B, Table B1). Deaggregation occurred in plasma containing SGS at all concentrations of adrenalin above 1.0 μM (Fig. 14c). At lower concentrations, deaggregation occurred in 2 of 6 replicates in plasma containing SGS. However, the other 4 did not aggregate sufficiently. Deaggregation was not observed in controls, regardless of the adrenalin concentration (Fig. 14c).

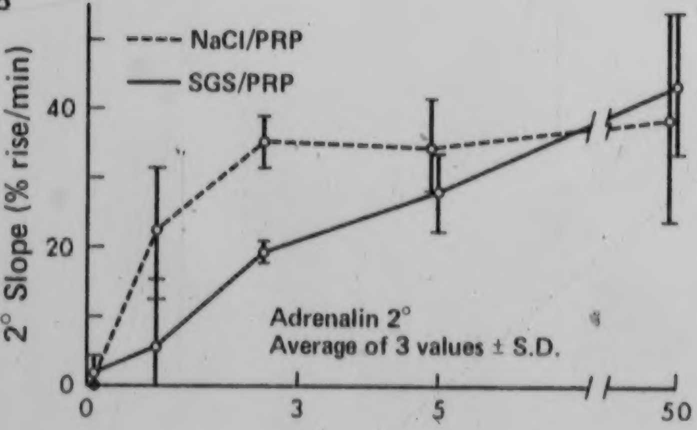
Figure 17. Primary slopes and maxima of aggregation curves induced by adrenalin .

Figure 18. Secondary slopes and maxima of aggregation curves induced by adrenalin .

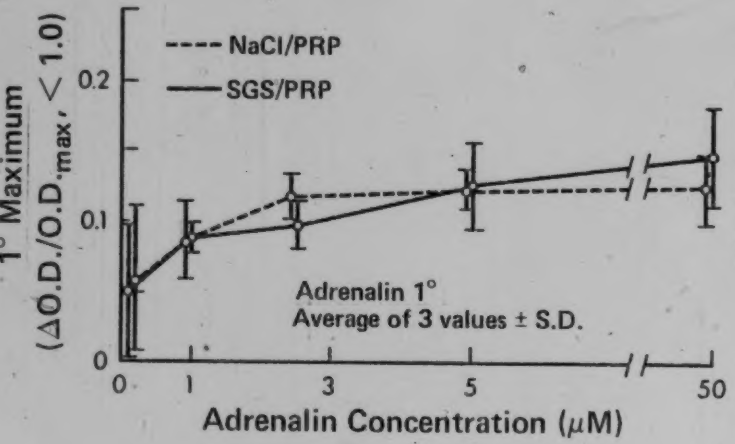
17


1° Slope (% rise/min)
0
10
20
---- NaCl/PRP
—— SGS/PRP
Adrenalin 1°
Average of 3 values ± S.D.
0
1
3
5
50

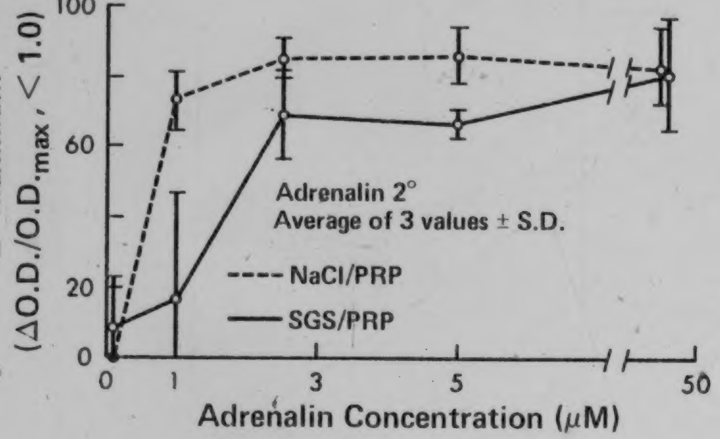
18


2° Slope (% rise/min)
0
20
40
---- NaCl/PRP
—— SGS/PRP
Adrenalin 2°
Average of 3 values ± S.D.
0
3
5
50

17


1° Maximum
(ΔO.D./O.D.max, < 1.0)
0
0.1
0.2
---- NaCl/PRP
—— SGS/PRP
Adrenalin 1°
Average of 3 values ± S.D.
0
1
3
5
50
Adrenalin Concentration (μM)

18


2° Maximum
(ΔO.D./O.D.max, < 1.0)
0
20
60
100
Adrenalin 2°
Average of 3 values ± S.D.
---- NaCl/PRP
—— SGS/PRP
0
1
3
5
50
Adrenalin Concentration (μM)

Ristocetin-induced aggregation: Ristocetin-induced platelet aggregation was not significantly affected in plasma containing SGS (Fig. 14d, 19; Appendix B, Table B3, B4), although maxima at 1.5 mg/ml and the slope at 1.2 mg/ml had means and standard deviations different from controls (Fig. 19).

Thrombin-induced aggregation: Thrombin-induced platelet aggregation was completely abolished in plasma containing SGS (Fig. 14e, 20). Final concentration of 10 NIH unit/ml thrombin failed to induce platelet aggregation. Although statistically (Appendix B, Table B3, B4) slopes and maxima of plasma containing SGS were inhibited, (dependent on thrombin concentration) valid statistical analysis is difficult, since control plasma clotted at thrombin concentrations above 0.25 NIH unit/ml.

2.4.4.3 Scanning electron microscopy of platelets

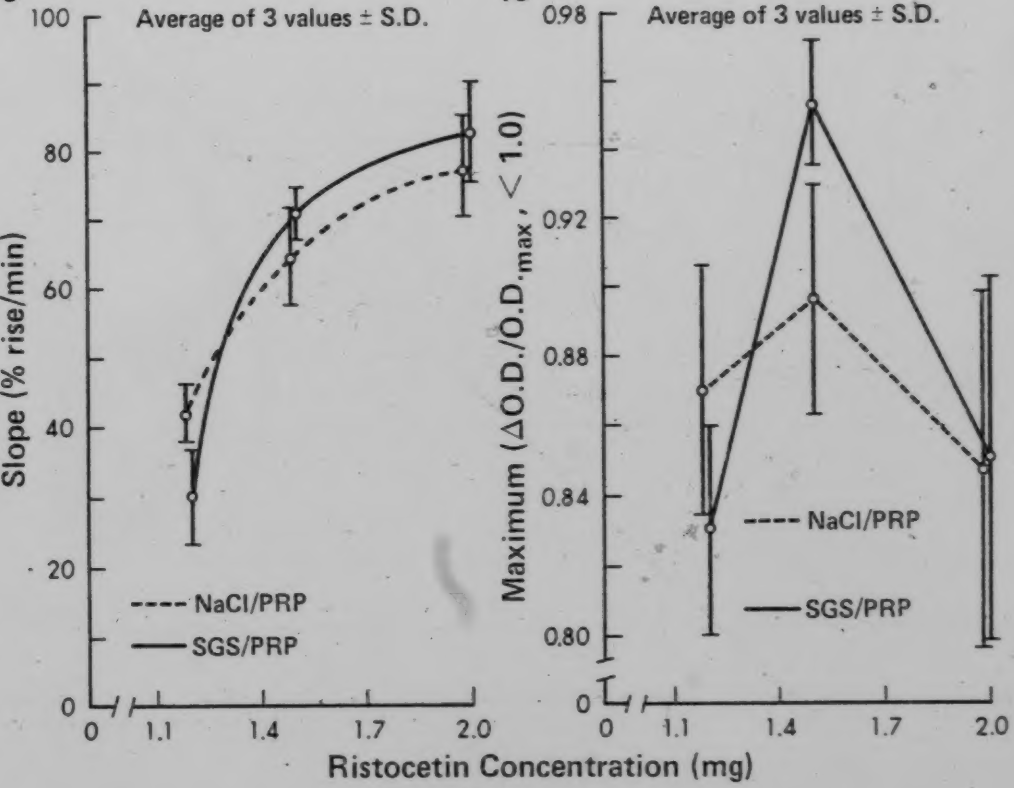
SEM: Controls: After 5 minutes stirring at 37°C, platelets from NaCl/PRP (20 platelets were observed) were mostly ovoid or slightly spherical, although some retained their native disc shape. Most platelets developed pseudopodia numbering less than 4 and had a smooth, but irregular surface contour (Fig. 21). Most remained single, although some unfused clumps of 3 to 7 platelets were observed. These platelets appeared to be similar to those described by Hovig (1970) and Walsh and Barnhart (1973). Platelets obtained from SGS/PRP (28 were observed) were similar to NaCl/PRP (Fig. 22), indicating that SGS has no detectable effect on platelet surface structure.

SEM: Acid collagen (1:500): In the presence of acid collagen, platelets from NaCl/PRP lost their individual platelet contour and developed large aggregates (2 were observed) with a 'coral' like appearance,

Figure 19. Slopes and maxima of aggregation curves induced by ristocetin.

Figure 20. Slopes and maxima of aggregation curves induced by thrombin. Maxima at 1.0 NIH unit/ml final concentration in PRP is omitted due to clot artifact.

19


Average of 3 values ± S.D.
Slope (% rise/min)
100
80
60
40
20
0
NaCl/PRP
SGS/PRP
Average of 3 values ± S.D.
Maximum (ΔO.D./O.D.max, < 1.0)
0.98
0.92
0.88
0.84
0.80
0
NaCl/PRP
SGS/PRP
0
1.1
1.4
1.7
2.0
Ristocetin Concentration (mg)

20

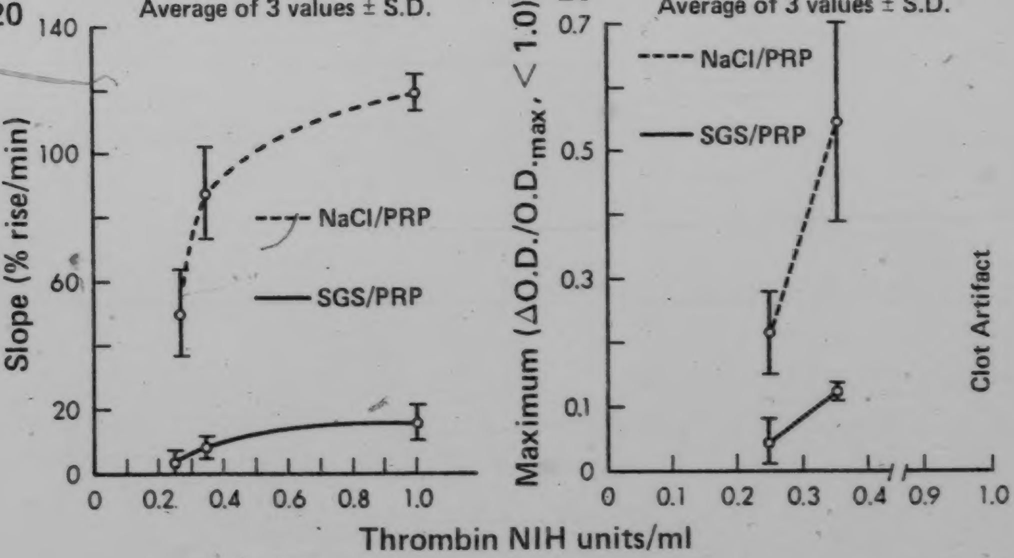

Average of 3 values ± S.D.
Slope (% rise/min)
140
100
60
20
0
NaCl/PRP
SGS/PRP
0
0.2
0.4
0.6
0.8
1.0
Average of 3 values ± S.D.
Maximum (ΔO.D./O.D.max, < 1.0)
0.7
0.5
0.3
0.1
0
NaCl/PRP
SGS/PRP
Clot Artifact
0
0.1
0.2
0.3
0.4
0.9
1.0
Thrombin NIH units/ml

Figure 21. SEM of platlet from NaCl/PRP stirred for 5 minutes at 37^{o}C. Note ovoid shape and smooth, though irregular surface contour.

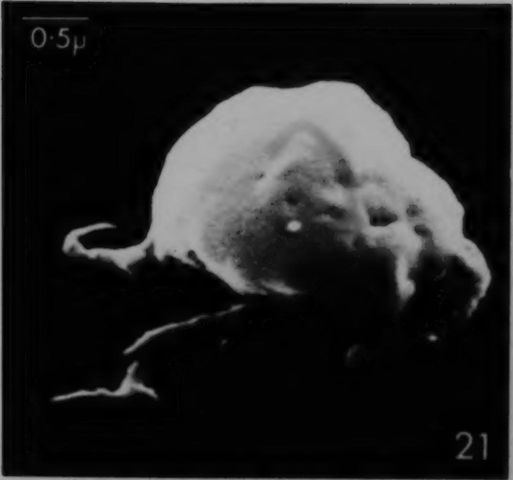
Figure 22. SEM of platelet from SGS/PRP stirred for 5 minutes at 37^{o}C. Note SGS has no detectable effect on platelet surface structure (same as Fig. 21).

Figure 23. SEM of platelet aggregate from NaCl/PRP following aggregation induced by 1:500 acid collagen. Close up of Fig. 24. Note 'coral' like appearance.

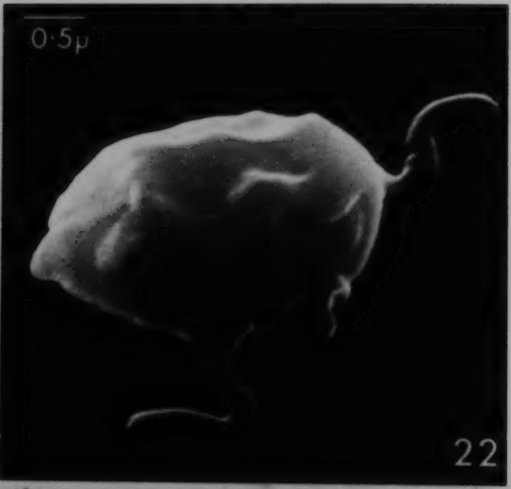
Figure 24. SEM of platelet aggregate from NaCl/PRP following aggregation induced by 1:500 acid collagen.

Figure 25. SEM of platelets from SGS/PRP following aggregation induced by 1:500 acid collagen. Note no aggregates and difference from control in Fig. 23 and 24. Pseudopodia appear unfused to adjacent platelets.

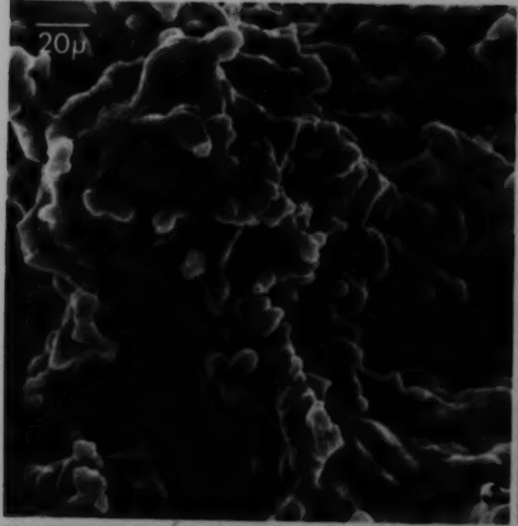
Figure 26. SEM of platelet from SGS/PRP following aggregation induced by 0.35 NIH unit/ml thrombin. Controls clotted, but have been reported similar to Fig. 27. Platelet surface is smooth. No aggregates were observed.

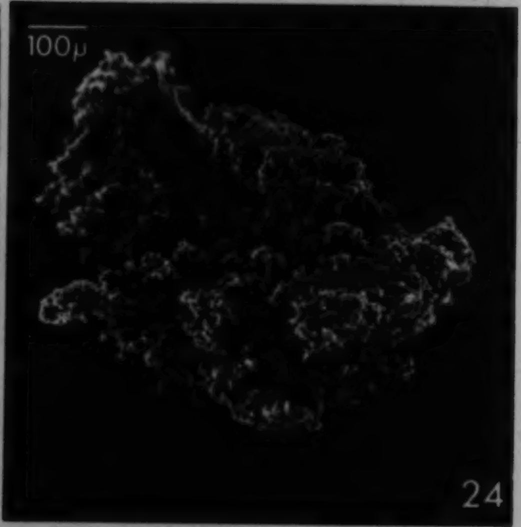


21

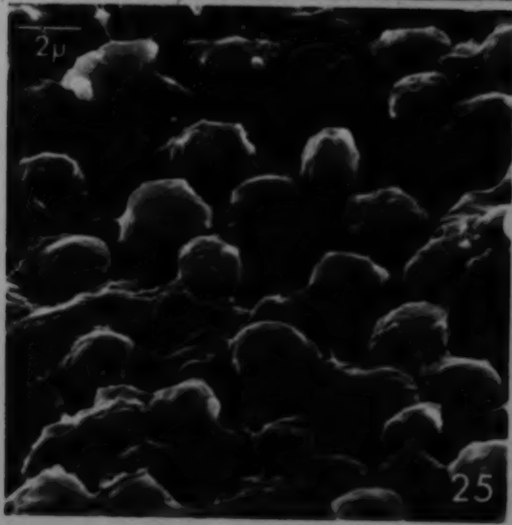


22

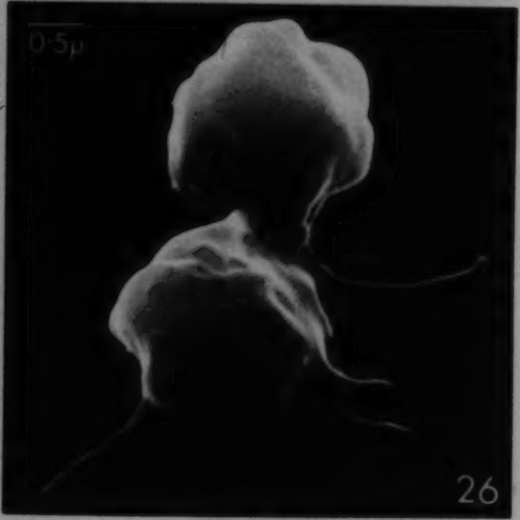




24



25



26

similar to those described by Larrimer et al. (1970) (Fig. 23,24). Platelets from SGS/PRP (more than 50 were observed) remained single, and developed numerous pseudopodia which did not appear fused with adjacent platelets (Fig. 25).

SEM: Thrombin (0.35 NIH unit/ml): Clot formation precluded examination of the effect of thrombin on platelets in NaCl/PRP. Thrombin (0.1 unit/ml) normally causes formation of large aggregates and an irregular platelet surface contour similar to ADP (Larrimer et al., 1970). In the presence of SGS, these normal thrombin induced changes did not occur (Fig. 26). Platelets (13 were observed) were single, disc shaped, smooth and developed few pseudopodia.

SEM: ADP (1 μM): In the presence of ADP, platelets in NaCl/PRP formed large aggregates, became spherical, sometimes with surface convolution, and developed numerous pseudopodia (Fig. 27, 28; 2 aggregates and 10 single platelets were observed). These platelets were similar to those described by Hovig (1970), Clarke et al. (1969) and Walsh and Barnhart (1973). When SGS was also present, platelet aggregates were absent, sphering and surface convolution did not occur, and the number of pseudopodia was less (Fig. 29, 30; 36 platelets were observed).

SEM: Adrenalin (5 μM): Platelets from SGS/PRP and NaCl/PRP following the addition of adrenalin were similar. Large aggregates with peripherally visible individual platelet contour (Fig. 31, 32) found in both samples, were similar to those described by Larrimer et al. (1970).

2.4.5 Effect of naturally produced rabbit antibodies to tsetse on anticoagulant activity

Naturally produced antibodies to tsetse had no effect on the anticoagulant activity of SGS (Table 8).

Figure 27. SEM of platelet from NaCl/PRP following aggregation induced by 1 μM ADP. Note rough surface contour in comparison to Figure 26.

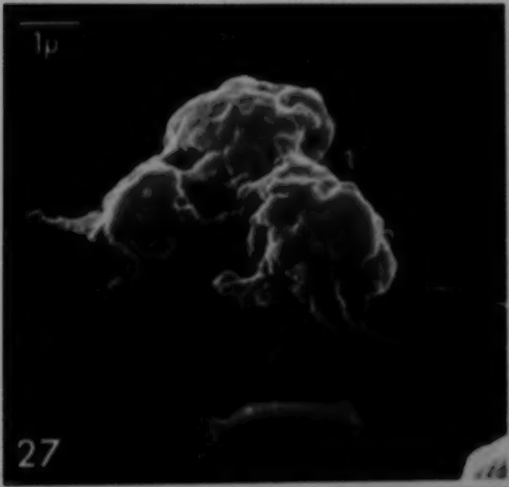
Figure 28. SEM of platelet from SGS/PRP following aggregation induced by 1 μM ADP. Note smooth platelet surface and similarity to Figures 21 and 22.

Figure 29. SEM of platelet aggregate from NaCl/PRP following aggregation induced by 1 μM ADP. Note difference from Fig. 30.

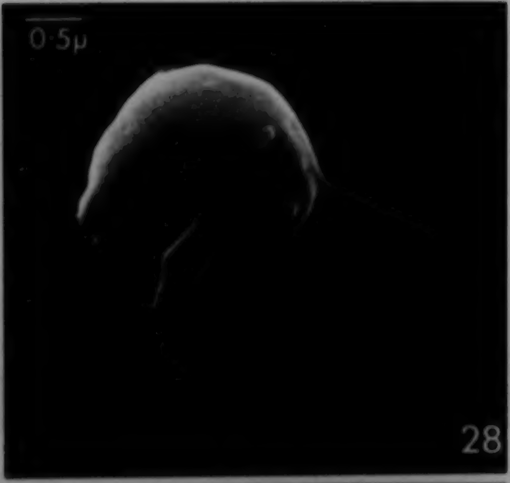
Figure 30. SEM of platelets from SGS/PRP following aggregation induced by 1 μM ADP. Note no aggregates. Platelets are unfused and lack the rough surface contour of those in Figure 27.

Figure 31. SEM of platelet aggregate from NaCl/PRP following aggregation induced by 5 μM adrenalin . Note similarity with Figure 32.

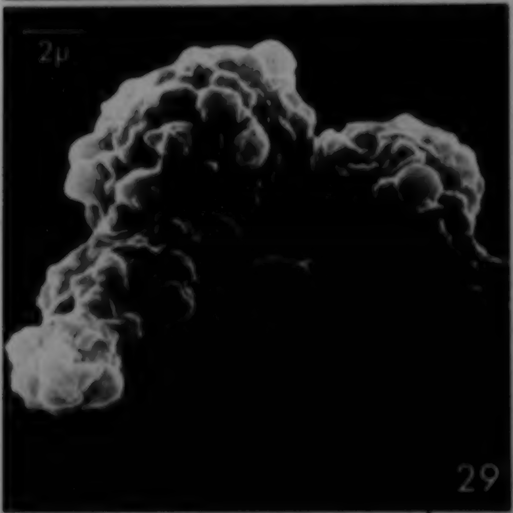
Figure 32. SEM of platelet aggregate from SGS/PRP following aggregation induced by 5 μM adrenalin .



27



28



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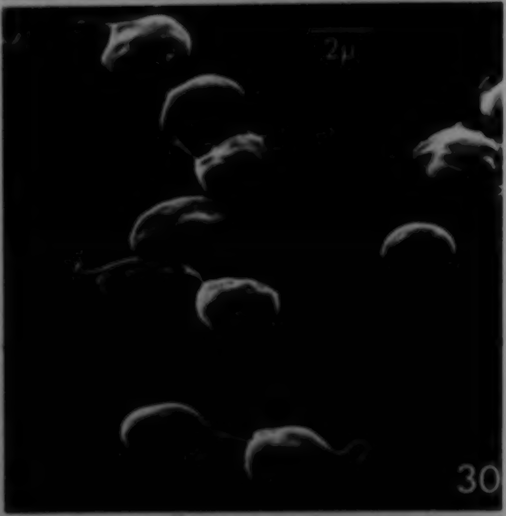
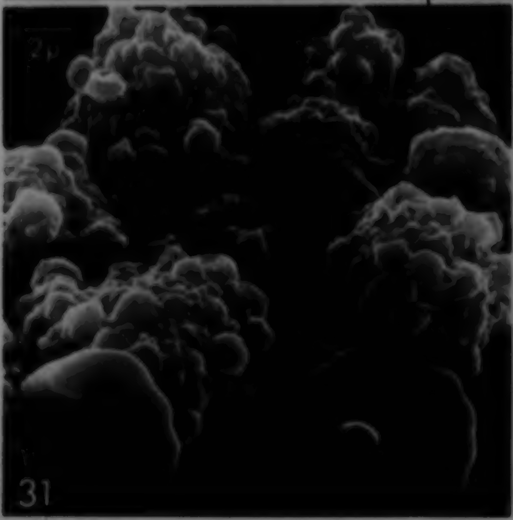
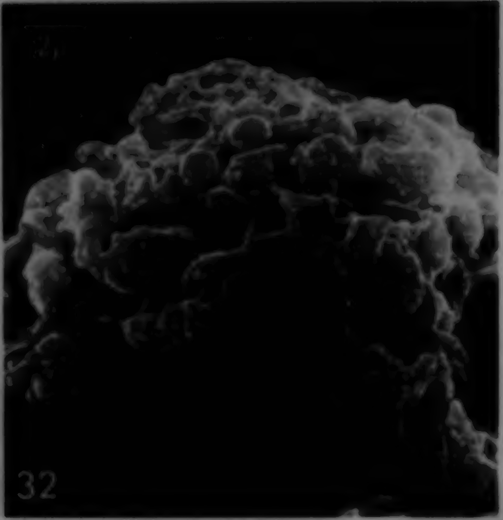


30



31



32

Table 8. Effect of naturally produced rabbit antibodies to SGS on anticoagulant activity.

Serum Rabbit no.	Date	Antibody[a] titre	Clotting time (sec) of control (NaCl/CPP) and test (SGS/CPP)[b] plasma (0.85 ml) following 40 minutes incubation with serum (0.15 ml) PT Control	PT Test	TT Control	TT Test	APTT Control	APTT Test
Control serum								
3PD8	June 3/75	0	12.8	19.8	10.1	>300	52.5	128.0
3PD1	Jan. 27/75	0	12.8	19.3	9.6	>300	52.5	129.0
Test serum								
3PD8	Dec. 7/75	+32,000	12.3	19.6	9.8	>300	50.2	113.7
3PD1	Dec. 7/75	+ 8,192	12.8	20.6	10.3	>300	56.6	121.5

[a] Antibody titres determined by passive haemagglutination technique (see Section 3.4.2).

[b] Clotting times are averages of duplicates.

2.5 Discussion

The anticoagulant of G. austeni was reported to be an antithrombin (Hawkins, 1966). However, the experimental techniques used did not conclusively demonstrate that an antithrombin was present, nor did they preclude additional anticoagulant activity. In the experiments reported here, G. morsitans saliva was found to prolong the PT, TT and the APTT. Its action was immediate. The results of clotting factor assays in the presence of SGS excluded a specific inhibitory effect on clotting factors II, V, VII, VIII, IX, X, XI or XII, similar to the anti-factor VIII activity reported in the salivary glands of Rhodnius prolixus (Hellmann and Hawkins, 1965) and the anti-factor IX activity reported in the salivary glands of Ornithodorus moubata (Hellmann and Hawkins, 1967). The lack of effect of SGS on the reptilase time excludes inhibition of the conversion of fibrinogen to fibrin and interference with fibrin polymerization (Latallo and Teisseyre, 1971). Thus, using detailed studies the anticoagulant of G. morsitans has been shown to be an antithrombin alone.

G. morsitans anticoagulant activity has an effect similar to, but a mechanism different from heparin. Both are antithrombins which prolong the PT, TT and the APTT and have similar molecular weights (Heparin 16,000 : Seegers, 1967; SGS 11,000 to 13,000). Unlike heparin, the anticoagulant activity of SGS is not neutralized by toluidine blue or protamine sulphate and does not require Antithrombin III for maximal prolongation of the clotting time. These results demonstrate that the anticoagulant properties are not due to heparin, and substantiate similar results based on histochemical analysis (Fairbairn and Williamson, 1956). The properties of SGS are similar to hirudin. Both are antithrombins, heat stable, inhibit thrombin hydrolysis of TAME, do not require Antithrombin III for inhibition of coagulation, and inhibit platelet aggregation (completely when induced by thrombin) (Markwardt, 1961, 1963; Seegers, 1967). The only property which differentiates the two, is that Sephadex G-75 fractionation of hirudin results in a protein peak (A_{280}) corresponding with anticoagulant activity (Bagdy et al., 1973), whereas SGS does not. Further biochemical analysis of SGS will probably confirm a difference, although such analysis was not within the scope of this study.

In contrast to reports that the salivary gland activity of G. tachinoides and G. morsitans is destroyed after 30 minutes heating at 100°C (Lester and Lloyd, 1928), in the present study, the G. morsitans anticoagulant, purified by Sephadex gel filtration, was heat stable. The reason for the increase in the TT but not in the APTT with prolonged heating remains unexplained. Anticoagulant activity of SGS remained stable in aqueous solution for at least 1 month at +4° and -20°C without the addition of antibacterial agents. Tsetse anticoagulant activity was not neutralized by human anti-Antithrombin III serum. Sephadex fractionation of SGS produced a single peak of anticoagulant activity corresponding to a molecular weight of 11,000 to 13,000 confirming the prediction of a low molecular weight by Fairbairn and Williamson (1956). Maximum anticoagulant activity did not correspond with absorbance at 280 nm readings, indicating that it is probably non-proteinaceous although not excluding low protein content or a protein potent in minute quantities. Inhibition of thrombin hydrolysis of TAME in the presence of SGS, and its correlation in Sephadex fractions with maximum anticoagulant activity, indicate that the esterolytic site as well as the proteolytic site of thrombin is inhibited. The antithrombins, hirudin, reduviin, but not tabanin, inhibit thrombin hydrolysis of TAME, although, as with G. morsitans SGS, hirudin does not completely inhibit esterolytic activity (Markwardt, 1961).

Although a plasminogen activator has been reported in the salivary glands of G. austeni (Hawkins, 1966), I was unable to identify either a fibrinolysin or a plasminogen activator in G. morsitans, despite the fact that I used 10 to 20 times the number of glands used by Hawkins (1966: 0.1 ml of 0.9% NaCl/insect). In both studies, fibrinolytic activity was identified in fly gut tissue. Hawkins removed both salivary glands and gut tissue while dissecting flies, thus her salivary gland preparations may have been contaminated by gut tissue activity. The interesting possibility also exists that G. morsitans and G. austeni, two closely related species, have salivary glands which differ in some of their properties.

Yorke and MacFie (1924) reported there was no haemolytic agent in the salivary glands of G. tachinoides. My results, using G. morsitans SGS and rabbit erythrocytes are in agreement. The function of the incubated osmotic fragility test is to measure changes in erythrocyte fragility. Its modified use for the detection of slow reacting haemolytic substances, such as may be contained in arthropod salivary glands and whose function may be to aid in digestion of the blood meal, does not appear to have been previously described. Further modified use of this test may demonstrate the presence of substances which increase erythrocyte osmotic fragility, but do not lyse erythrocytes and therefore remain undetected by other methods presently in use. Comparable with other techniques, the incubated osmotic fragility test can detect the presence of strong haemolytic substances which do lyse erythrocytes, as demonstrated with the digestive enzymes contained in G. morsitans posterior midgut tissue.

Although platelets are an integral part of normal haemostasis, the effect of insect secretions on their function does not appear to have been previously described. In platelet-rich plasma containing SGS, adherence of platelets to collagen was not affected. However, platelet aggregation was inhibited, depending on the type and concentration of the aggregating agent used. G. morsitans SGS did not induce spontaneous aggregation in normal platelet-rich plasma. Thrombin-induced platelet aggregation was completely inhibited in the presence of SGS. This phenomenon is probably due to the antithrombin activity of SGS rather than an effect on a platelet receptor site. Recent evidence indicates that thrombin binds to the platelet membrane at a site different from its proteolytic site (review: Cooper et al., 1976). Investigations with hirudin suggest that it acts on thrombin through binding the cationic groups of thrombin which bind the fibrinopeptide region of the fibrinogen substrate (Markwardt, 1970). Since hirudin inhibits the binding of thrombin to platelets (Ganguly and Sonnichsen, 1976) and inhibits the thrombin-induced release reaction (Detwiler and Feinman, 1973), Ganguly and Sonnichsen (1976) suggest that the platelet receptor recognizes that part of the thrombin molecule which also binds to fibrinogen. Furthermore, recent studies have demonstrated that fibrinogen located on the platelet surface is not the receptor of thrombin (Tollefsen and Majerus, 1975). In view of this information, Ganguly and Sonnichsen (1976) suggest, that since thrombin, with its active serine site blocked, will still bind to platelets but will not cause aggregation and release (Ganguly, 1974; Tollefsen et al., 1974), then both the binding site and the active site of thrombin are necessary for the action of thrombin on platelets. Similarly, the inhibition of SGS on thrombin-induced platelet aggregation may be the result of the antithrombin having blocked the proteolytic and esterolytic sites on the thrombin molecule rather than on a specific platelet receptor site. It is also possible, since both hirudin and SGS inhibit thrombin-induced release and have a number of similar effects on the haemostatic mechanism, that the action of SGS on thrombin is similar to Markwardt's suggested action for hirudin.

Most aggregating agents are believed to function through inducing release of endogenous platelet ADP (reviews: Mustard and Packham, 1970, 1975). The aggregation curves obtained using various aggregants in the presence of SGS cannot be simply explained as inhibiting this release mechanism. The secondary wave of ADP-induced platelet aggregation was completely abolished by SGS, indicating that the release of platelet constituents is inhibited. However, reason for abolition of secondary wave ADP, but only partial inhibition followed by deaggregation of secondary wave adrenalin - induced platelet aggregation remains uncertain, since both phases involve release of endogenous ADP (see Williams et al., 1972). Similarly, acid collagen, which induces a single phase platelet response through the release of endogenous ADP (review by Mustard and Packham, 1970), was only partially inhibited by SGS. Ristocetin-induced platelet aggregation, in which the second, but not the first phase is associated with the release reaction (Cazenave et al., 1973b), was not significantly affected in the presence of SGS. The inhibitory action of SGS to inhibit platelet release would thus appear to be of complex nature. However, recent investigations on the mechanisms of platelet aggregation and the subsequent role of thrombin indicate that the antithrombin action of SGS may be responsible for all of these platelet inhibitory effects.

Ardlie and Han (1974) have recently demonstrated that aggregation induced by ADP is mediated initially by thrombin-fibrinogen complexes between adjacent adhering platelets. The thrombin generated on the platelet membrane, through the interaction of clotting factors, aids in fibrin polymerization and ultimately causes the release of platelet constituents and irreversible aggregation. The inhibitory effect of SGS on ADP-induced platelet aggregation may be explained by the antithrombin inhibiting this action of thrombin. In support of this possibility, SGS inhibited the second wave of ADP-induced platelet aggregation (release of platelet constituents) and resulted in deaggregation at all concentrations of ADP in comparison to deaggregation in NaCl controls only at optimal ADP concentration. Furthermore, SEM of platelets from plasma containing SGS, following the addition of ADP, were not similar to saline controls induced to release under the same conditions, but were similar to unstimulated platelets. The inhibitory effects of SGS on ADP-induced platelet aggregation may therefore be the result of the antithrombin of SGS inhibiting thrombin-fibrin polymerization rather than reacting with a specific receptor site. Similar to SGS, heparin inhibits the second phase of ADP-induced platelet aggregation and release but does not inhibit the first phase of aggregation (Ardlie and Han, 1974). Consequently, and similar to the action proposed for heparin (Ardlie and Han, 1974), trace amounts of thrombin normally associated with platelets account for the initial reversible interaction between platelets upon the addition of ADP, and that subsequent inhibition of thrombin formation on the platelet surface by the antithrombin action of SGS accounts for the lack of irreversible aggregation and the inhibition of release of platelet constituents.

The antithrombin of SGS may also be responsible for the inhibitory effects observed in collagen-induced platelet aggregation. Collagen has recently been shown to induce release of platelet constituents by two different mechanisms; an indirect mechanism, dependent on blood coagulation factors via the intrinsic pathway, and a direct mechanism independent of factor XII, but requiring factor XI (Huzoor-Akbar and Ardlie, 1976). Furthermore, heparin and hirudin inhibit the indirect mechanism but have no effect on the direct mechanism. Huzoor-Akbar and Ardlie (1976) suggest that collagen-induced release of platelet constituents is, in part, due to a direct effect on the platelet, and in part, due to an indirect effect involving coagulation factors and mediated by thrombin. They further conclude that irreversible aggregation by collagen is mediated by thrombin. In plasma containing SGS, slopes and maxima of aggregation induced by collagen were inhibited. There was no indication of deaggregation. These results may be explained by the antithrombin of SGS inhibiting thrombin's participation in the indirect mechanism, similar to the inhibition induced by heparin and hirudin. Aggregation, induced by collagen in the presence of SGS, but only at an inhibited rate, may be explained by the direct mechanism of collagen on the platelets. Lack of deaggregation in plasma containing SGS could result from sufficient release of platelet constituents via the direct mechanism to further stimulate other platelets.

The second wave of aggregation and the release reaction induced by adrenalin requires (as does aggregation induced by ADP) one or more plasma factors in addition to fibrinogen (Cronberg et al., 1970; Bang et al., 1970). Although thrombin has apparently not been demonstrated as playing a role in adrenalin-induced platelet aggregation, the inhibition of second phase aggregation and subsequent deaggregation resulting from the presence of SGS in platelet-rich plasma may be due to the antithrombin affecting aggregation and release, similar to the inhibition of ADP-induced platelet aggregation.

Further evidence that the inhibitory effects of SGS in plasma induced to aggregate with various agents are probably due solely to the action of the antithrombin, come from the results of ristocetin-induced platelet aggregation. Although the precise mechanism of ristocetin-induced platelet aggregation remains to be clarified (Jenkins et al., 1975), there is evidence that ristocetin binds to the platelet membrane (Kattlove and Gomez, 1975). Since ristocetin does not require fibrinogen as a co-factor to induce aggregation (Jenkins et al., 1974) and in fact precipitates fibrinogen from plasma (Howard and Firkin, 1971), it is unlikely that thrombin-fibrinogen complexes are involved. Consequently, since ristocetin-induced platelet aggregation was not inhibited in the presence of SGS, it is supportive evidence that the antithrombin of SGS acts on thrombin's participation in aggregation rather than on a specific platelet receptor site.

Scanning electron microscopy of platelets following aggregation gave results consistent with platelet aggregation studies. Addition of SGS to platelet-rich plasma induced no change in platelet surface structure. Most platelets from plasma containing SGS, after addition of acid collagen, ADP and thrombin, showed little change from the surface structure of unstimulated platelets. Following the addition of aggregating agents to control plasma, platelet changes were similar to those of previous authors (Clarke et al., 1969; Hovig, 1970; Larrimer et al., 1970; Walsh and Barnhart, 1973).

Galun and Rice (1971) reported that platelets, rather than erythrocytes, may be the haematogustatory stimulus inducing gorging in blood-sucking insects. Results indicated that Aedes aegypti and G. morsitans feed more frequently through artificial membranes on solutions of erythrocytes and platelets, than erythrocytes alone. These authors suggested that only a local response at the chemoreceptor surface (possibly with ATP) is necessary to obtain initial feeding stimuli. Intact erythrocytes and dissolute (not referred to as lysed) platelets in the oesophagous of these flies supported their suggestion. If this were true, one would expect that either the salivary gland solution induces a release mechanism in the platelet, near the region of the chemoreceptors of the labellum, or that release of adenine nucleotides and other contents from platelets is caused by platelet interaction with the foreign surface of the mouthparts. Platelets are known to release among other substances, adenine nucleotides, particularly ADP and ATP, potassium, serotonin, histamine, and epinephrine (review: Mustard and Packham, 1970). Furthermore, labellar sensilla of the tsetse are known to respond to adenine nucleotides (Mitchell, 1976a, 1976b; Mitchell and Reinhouts Van Haga-Kelker, 1976). In my experiments, failure of SGS to induce spontaneous aggregation, the inhibitory effect of SGS in the presence of all aggregating agents except ristocetin, and inhibition of endogenous release of ADP, do not support the possibility that tsetse salivary components induce a release mechanism in the platelet resulting in a haematogustatory response. It is possible however, that interaction of the platelets with fly mouthparts may induce the platelets to adhere and release, thus producing localized concentrations of ADP and eliciting the haematogustatory response.

Prostaglandin E_2 has recently been reported in the salivary glands of Boophilus microplus (Higgs et al., 1976). Although this prostaglandin is discussed for its possible role in increasing vasodilation and blood vessel permeability, it is also known to affect platelet function, particularly aggregation (Kloeze, 1969; Shio and Ramwell, 1972; Salzman et al., 1972; Malmsten et al., 1975). Other reports of esterases in the salivary glands of this species (Geczy et al., 1971), and their suggested role in initiation and maintenance of the lesion of the host, tend to indicate that the function of the salivary components is more complex than has been previously described.

Cornwall and Patton (1914) reported that sera from a rat or rabbit hyperimmunized with salivary tissues of Philaematomya insignis partially inhibited the ability of anticoagulant from these glands to prevent clotting of whole blood. In contrast, immune serum from a dog refractory to further infections of Ancylostoma caninum did not affect the prothrombin time in the presence of amphidial gland extracts (Thorson, 1956). In my experiments, rabbit sera containing high antibody titres to naturally injected tsetse salivary solution failed to influence the anticoagulant activity of SGS. Thus, it appears that the anticoagulant in G. morsitans is not antigenic, at least when naturally injected into rabbits.

CHAPTER 3

HOST RESPONSE TO TSETSE EXPOSURE

3.1. Introduction

In a recent comprehensive review, Nelson et al. (1977) summarized the current status of the literature on host-ectoparasite interactions. This introduction therefore, will serve only to acquaint the reader with some of the major points discussed in their review, placing emphasis on research concerning biting-flies, the factors contributing to the development of host-resistance, and any recent publications.

Biting-flies cause considerable economic losses to the livestock industry, primarily through the disease organisms they transmit, direct mechanical damage, and annoyance (review by Steelman, 1976). Consequently, reports of decreases in productivity are common (Tashiro and Schwardt, 1949; review by Steelman, 1976; Campbell, 1976; review by Nelson et al., 1977). Anemia has also been frequently reported in host response to fleas, mites, ticks, and lice (see review by Nelson et al., 1977), however, comparable reports with biting-flies are lacking. Some authors have attempted to estimate blood losses in cattle using fly exposure and blood-meal size (Webb and Wells, 1924; Philip, 1931; Tashiro and Schwardt, 1949) however, concurrent examination of host-haematological parameters were not used to substantiate results. As pointed out by Steelman (1976), this type of information is essential, but unfortunately not available, in determining which levels of biting-fly attacks warrant control measures. Whiting et al. (1954) were unable to report significant signs of anemia in sheep exposed to low levels (100 to 500) of the ked, *Melophagus ovinus*, however, at levels of 2,000 keds per animal, Nelson (unpbl., from review by Nelson et al., 1977) reports sub-clinical signs, and Gecheva (1972) reports serious anemia resulting in death.

Heavy infestations of ectoparasites have affected host-blood chemistry and metabolic processes (see review by Nelson et al., 1975, 1977). The most comprehensive study of this nature deals with the effect of the tick, *Boophilus microplus*, on cattle (O'Kelly and Seifert, 1970; O'Kelly et al., 1971). Comparable reports with biting-flies are lacking.

Most of the host's physiological responses to ectoparasites involve mechanisms important in the development of resistance. The feeding mechanism and type of mouthparts of haematophagous insects are of prime importance in determining this type of response (see review by Nelson et al., 1975, 1977). Most studies on feeding mechanisms of biting-flies, using either histological techniques or transillumination, indicated ectoparasites are pool feeders (telmophages- hippoboscid, *Lipoptena cervi*, Haarlov, 1965; *Glossina morsitans*, *Chrysops*, Gordon and Crewe, 1948; *Stomoxys* and the black gnat, *Leptoconops torrens*, Lavoipierre, 1965). However, mosquitoes were described as both vessel and pool feeders (Gordon and Lumsden, 1939) and the sheep ked, *Melophagus ovinus*, exclusively as a vessel feeder (solenophage- Nelson and Petrunia, 1969). Many authors have examined host cutaneous responses, both clinically and histopathologically (Horsfall, 1962; reviews: Shulman, 1967; Nelson et al., 1977). In response to injected salivary gland secretions, host reactions have been classified in a series of responses known as the five-stage sequence of reactivity (Mellanby, 1944). In review of the stages which occur in hosts as a result of feeding of different ectoparasites, Nelson et al. (1977) attribute the differences between host response to ticks and mites and the response to mosquitoes and fleas as a function of the feeding mechanism.

One of the major problems involved in studying host development of resistance is deciding which factors indicate resistance. A number of factors including host-nutritional status, sex, age, and blood-chemistry levels are known to affect parasite populations and must be controlled prior to establishing levels of resistance. Authors have erroneously described possible resistance in hosts when other subtle factors, such as intraspecific ectoparasite competition, have naturally reduced parasite populations (see review by Nelson et al., 1977).

Acquired host-resistance has been examined in two host-ectoparasite systems, the louse-mouse system including at different times Polyplax serrata (Bell et al., 1966; Nelson et al., 1972; Nelson, unpubl., from review by Nelson et al., 1977) and Haematopinus eurysternus (Haufe et al., unpubl., from review by Nelson et al., 1977; Nelson, pers. commun.), and the ked-sheep system using Melophagus ovinus (Nelson and Bainborough, 1963; Nelson, unpubl., from review by Nelson et al., 1977). Acquired resistance in both systems is described as local. The development of resistance affects parasites of both systems in a similar manner, although different mechanisms are thought to be involved. Ectoparasites increase in number from one or more focal points on the host. Through irritation caused by the feeding of the parasite, the host develops localized skin-resistance, and the parasite in response, moves out radially to areas of less resistance. Restricted by some unfavourable sites on the host, such as the backs of grazing sheep (but not backs of sheep in winter or indoors), the parasite is gradually limited in movement and reduced in number (Nelson, pers. commun.). Nelson suggests that chronic irritation plays a substantial role in the development of resistance, however the extent and the mechanisms involved are not yet known. Although both the ked and the sucking-lice are solenophages, the prestomal teeth used by the lice may play a greater role in initiating irritation since the prestomal teeth of the sheep-ked act at the vessel level (Nelson and Petrunia, 1969), while the prestomal teeth of the louse act at the epidermal level (Lavoipierre, 1967). Histological studies with resistant (CFW) and susceptible (C57BL) mice exposed to lice have confirmed susceptibility is characterized by a lack of mast cells and polymorphonuclear neutrophilic leukocytes and an increase in polymorphonuclear eosinophilic leukocytes (Nelson and Bainborough, 1963; Nelson, unpubl., from review by Nelson et al., 1977 and pers. commun.). Although the mechanisms involved with the development of resistance are not known, Nelson suggests that a complement factor, now known to be involved with the tick saliva and neutrophils in tick bites (Berenberg et al., 1972), may be absent in susceptible mice (Nelson, pers. commun.). In the ked-sheep system, but not the louse-mouse system, fibrinoid degeneration of the arteriolar tunica media suggests an antibody-antigen complex is involved (Nelson and Bainborough, 1963; Nelson unpubl., from review by Nelson et al., 1977). Arteriolar vasoconstriction has been shown to be associated with acquired host-resistance in both the ked-sheep and the louse-mouse system (see review by Nelson et al., 1977).

Acquired resistance to ticks has been described as systemic rather than local (review: Nelson et al., 1977). Trager (1939a, 1939b) first suggested that hosts respond to the tick, Dermacentor variabilis through a 'walling off mechanism'. Hypersensitivity to *Boophilus microplus*, mediated by skin-sensitizing antibodies in cattle, may play a role in resistance (Riek, 1962). However, other authors believe that reactions involved with skin hypersensitivity contribute to host susceptibility (Tatchell and Moorhouse, 1968). The infiltration of basophils into lesions of cattle produced by *Dermacentor variabilis* larvae (Allen, 1973) and adult *Ixodes holocyclus* (Allen et al., 1977) provide evidence suggesting resistance is cell-mediated. Nelson et al. (1977) suggested that if these cells are ingested by the parasite, then enzymatic or immunologic mechanisms, rather than the proposed 'walling off mechanism', may be responsible for the decline in parasite populations. Mast cell disruption and infiltration and concentration of eosinophils in the lesions of cattle exposed to *Boophilus microplus* larvae are also presented as evidence that immune mechanisms are involved in resistance (Schleger et al., 1976). The infiltration of neutrophils in tick-bites on rats is reported to be stimulated by a chemoattractant which is formed when components of tick saliva bind with serum complement (C5), similar to the mechanisms believed involved with the Arthus reaction (Berenberg et al., 1972). Recently, with the use of immunosuppressants, Wikel and Allen (1976) have produced evidence of an immediate humoral component in guinea pigs exposed to *D. andersoni* larvae. These authors believe the humoral component is required along with delayed cell infiltration for the full expression of acquired resistance.

The possibility that immunological mechanisms are involved with resistance is further substantiated by the presence of circulating antibodies, although as pointed out by Nelson (see review by Nelson et al., 1977) there is little evidence to indicate a protective function.

Circulating antibodies, which appear to increase the destructive efficiency of neutrophils, correlate with acquired resistance in dermatophytes (Roberts, 1967). Naturally occurring precipitating antibodies have been frequently demonstrated in hosts exposed to various acarina (see review by Nelson et al., 1977). Haemagglutination titre tests for demonstrating sequential changes in antibody titres have also been used with some success (Brossard, 1976; review by Nelson et al., 1977) although titres in most cases have been very low.

In review of the literature, Nelson et al. (1977) point out that only twice have naturally produced precipitating antibodies been demonstrated in hosts exposed to arthropods other than the acarina. One to three precipitin bands have been demonstrated with sera of rabbits and guinea pigs exposed to *Aedes aegypti* (Wilson and Clements, 1965) and with sera of rabbits exposed to *Rhodnius prolixus* (Fox and Bayona, 1968). Haemagglutination titre tests have been successfully used to determine changes in antibody of cattle to larval *Hypoderma* (Robertson, 1964; Boulard and Weintraub, 1973) and antibody of sheep infested with *Melophagus ovinus* (Robertson and Nelson, unpubl., from review by Nelson et al., 1977). Using immunofluorescent antibody techniques, antibody against *M. ovinus* has been demonstrated in the cells of the skin of sheep infested with this species (Nelson and Petrunia, unpubl., from review by Nelson et al., 1977). With the same technique, rabbit immunoglobulins on various tissues of *Sarcophaga falculata* have been demonstrated in this fly when fed serum from rabbits immunized with the appropriate fly tissue (Schlein et al., 1976). Changes in the lymph nodes of mice and hamsters characteristic of antibody production and to a lesser extent cell-mediated immune responses have been demonstrated following primary exposure of 20 *Aedes aegypti* bites (Mellink and Vos , 1977).

3.2 Scope of this research

Experimental studies with ectoparasites have been slow to elucidate mechanisms involved in the development of resistance. This has been due primarily to the complexity of the host mechanisms involved and the number of specialized fields required to study such a problem. Experimental studies concerning host response to biting-flies have been even further neglected because of the difficulties encountered in assessing and controlling biting-fly exposure. This laboratory model was designed to reduce these difficulties and provide information on host response to the rapid, intermittent type of feeding used by biting-flies. The advantages of pursuing this problem have been outlined in Chapter 1.

Physiological parameters such as weight changes, haematocrits, plasma thrombin times and in some cases red and white cell counts and whole blood clotting times were used to detect abnormalities in host metabolism. Rabbit sera, collected at various times throughout tsetse exposure, was used to detect the presence of, and the changes in, naturally produced antibodies. Fly blood-meal weights and probings during feeding were used in an attempt to measure localized skin-resistance.

3.3 Materials and methods

3.3.1 Rabbit weights and haematological parameters

Rabbit weights (±2.5 g) were obtained using a Morris full capacity beam scale (Morris Scale Co., Portland, Oregon). Blood samples were obtained from the marginal artery of the left ear using 21 gauge needles and disposable syringes. Duplicate heparinized haematocrit capillary tubes (Pre Cal, Dade) were filled directly from the puncture wound following withdrawal of the needle, centrifuged at 3700 g for 4 minutes (IEC 927 centrifuge head) and read using a Critocap micro-haematocrit tube reader (Sherwood Med. Ind., Bridgeton, Mo.). Red and white blood cell counts were obtained with a Levy corpuscle counting chamber and Neubauer grid (C.A. Hausson and Son, Pa.) using standard techniques (Benson and Gunstream, 1970). Thrombin times (Fletcher et al., 1959) were performed at 37°C using 3 NIH unit/ml bovine thrombin (Parke-Davis) in saline and citrated rabbit plasma. Capillary (Benson and Gunstream, 1970) and tube (Lee and White, 1913) whole blood clotting times were performed at 17±1.3°C.

3.3.2 Immunological techniques

3.3.2.1 Preparation of antigen

Antigen containing 0.15 to 0.25 mg protein/ml of 0.85% NaCl (determined by method of Lowry et al., 1951) was prepared as described for SGS (see Section 2.3.1).

3.3.2.2 Precipitation ring tests

Precipitin ring tests were performed using the method of Campbell et al. (1970).

3.3.2.3 Immunodiffusion

Slides were prepared with 0.8% agarose (Sea-Kem) containing

0.146 M NaCl and 1/10,000 merthiolate in 0.1 M phosphate buffer at pH 7.0. Gels were incubated in a moist chamber at 37°C for 4 to 6 days, washed with saline, distilled H_2O, dried with filter paper, and stained with Amido Schwartz solution (Brewer et al., 1974). Plates were destained in a methanol-acetic acid-distilled H_2O (50:1:50) solution.

3.3.2.4 Immunoelectrophoresis

Immunoelectrophoresis was performed using veronal buffer at pH 8.2 and ionic strength of 0.05 by the method of Grabar and Williams (1955). A spot of bromophenol blue (Fisher) placed parallel to the antigen well and inside one of the cut, but unremoved gel troughs, was used as a marker. Agar gels (0.8% Difco agar or Sea-Kem agarose) were electrophoresed at 25 to 40 mA/250-350 V until the bromophenol blue marker was approximately 1 cm beyond the distal end of the serum trough. Gels were washed and stained as described for immunodiffusion.

3.3.2.5 Passive haemagglutination titre (PHT) tests

Passive haemagglutination titre tests (Campbell et al., 1970) were performed using sheep red blood cells (Swift's, Edmonton) treated with tannic acid (Fisher). Both sera samples and antigen were heat inactivated at 56°C for 30 minutes and absorbed with an equal volume of washed sheep red cells. All sera samples from a particular rabbit were measured on the same day and in the same experiment. Standard controls for non-specific reactions of the antiserum and normal rabbit serum diluent were performed with every sample. The highest dilution of antiserum to fully agglutinate cells was marked as the positive titre.

3.3.3 Experimental design

3.3.3.1 Rabbits exposed to tsetses 2 to 3 times a week

Twelve male rabbits from 3 litters were used. Equal numbers of control (to receive no exposure) and experimental (to be exposed) rabbits were obtained from each litter. Experimental rabbits were exposed to 300 to 500 tsetses per day, 2 or 3 times a week, for a period of 8 months. Rabbit weights, red and white blood cell counts, capillary and tube clotting times and haematocrits were examined at various times during the 8 month period and compared with values obtained at the same time from control rabbits. Immunoelectrophoresis and passive haemagglutination titre tests were performed on sera samples collected at monthly intervals from 4 of the 6 rabbits exposed to tsetses.

3.3.3.2 Rabbits exposed to tsetses 6 days a week

Two male rabbits from separate litters and one pair of rabbits from a third, were exposed daily to 250 to 500 tsetses, 6 days a week, for 20 weeks. Three control rabbits, one male from each of the litters from which experimental rabbits were obtained, were held without exposure to tsetse flies for 11 weeks. During the tenth week control male 6AD12 died, for no apparent reason and was replaced by a female litter mate, 6AD11. During the twelfth to fifteenth week, the previously unexposed control rabbits were exposed to 120 flies per day, for 6 days. Control and experimental rabbits were bled (10cc) once a week, on the evening of the day experimental rabbits received no exposure. Control rabbits were bled from weeks 5 to 20 only. Record of rabbit weight and haematocrit were collected weekly. Citrated rabbit plasma, also collected at weekly intervals, was used for thrombin clotting times at the end of the experiment. Immunoelectrophoresis was performed on sera samples of all 4 rabbits receiving exposure throughout the 20 week period. Passive haemagglutination titres were obtained with weekly sera samples from 3 of the 4 rabbits exposed throughout the 20 week period. The total number of flies exposed to each of the rabbits was recorded daily, and used for estimates of host blood loss.

3.3.3.3 Rabbits exposed to 1200 to 1500 tsetses in 4 hours

Ten rabbits, from 1 to 3 years of age, were used. Three of the rabbits were exposed to tsetses for the first time, while another 3 had received fly exposure prior to this experiment. The remaining 4 rabbits, all of which had received no previous exposure to tsetses, were used as controls, handled in the same manner as exposed rabbits, except that cages strapped to the back and ears, contained no flies. Flies were starved 48 hours prior to feeding. Only one rabbit was exposed on a particular day. Four cages of flies, placed on the shaved back and both ears of the selected rabbit, were changed at 5 minute intervals for a period of approximately 4 hours. Visual estimates of the number of flies containing blood were used to calculate the number of fly-bites. Although 12 to 15 hundred flies were applied, estimates on the number feeding were usually only 10 to 12 hundred. Rabbit weight, haematocrit, and blood samples were taken from all rabbits immediately prior to exposure (or control handling), again shortly after exposure (or control handling) and periodically for 18 to 30 days post-exposure.

3.3.4 Measurement of host-skin resistance with flies

In a recent review of the mechanisms involved with acquired host-resistance, Nelson et al. (1977) discuss localized vascular responses as playing a major role. These experiments were designed to determine if host vascular responses such as arteriolar vasoconstriction and skin-thickening are reflected in the efficiency with which a blood meal is obtained and the size of the blood meal.

3.3.4.1 Fly blood-meal weights

Unfed, 24 to 48 hour old teneral tsetses of both sexes were used in both experiments. Blood-meal weights were obtained by:

a) weighing individually marked vials and corks

b) weighing the same vials containing 1 tsetse

c) placing each fly into its corresponding individually marked Geigy-1 cage (3 x 2.5 x 2.5 cm)

d) exposing flies for a period of 10 minutes to a selected portion of a rabbit

e) replacing each fly back in its respective vial and weighing on a Mettler H20T balance within 5 to 7 minutes post-feeding.

Time was essential in post-feed weighings, since flies excrete water from the serum within a short period. Six flies were fed at one time and only those flies visually seen to contain blood were weighed after exposure. Flies which did not feed were not recorded and not reused.

Experiment 1: Fly blood-meal weights obtained from the right ear and shaved backs of previously-exposed and naive (previously non-exposed) rabbits.

Four pair of rabbits were used, matching pairs by sex, and where possible, age. One rabbit in each pair had received no previous tsetse exposure. The other rabbit in each pair had received various levels of tsetse exposure prior to this experiment (Table 9). Of the 6 flies fed at one time, three were fed on the same portion of both rabbits in a pair. Within a 9 day period, a total of 493 fly blood-meal weights were obtained.

Statistical analysis of experiment 1: Analysis of variance was performed using subprogram ANOVA from the SPSS (Statistical Package for Social Sciences; Nie et al., 1970) package available on the computer, Computing Services, University of Alberta. Analysis of variance was performed:

a) using only fly blood-meal weights from the right ear. The main

Table 9. History of tsetse exposure for previously-exposed rabbits used in fly blood-meal experiment 1.

Rabbit	Exposure to tsetses (days/wk, days of week)[a]				
	Months prior to experiment 1				
	1	2	3	4	5
3BE5	3 days/wk 1,2,3	2 days/wk 1,4	2 days/wk 1,4	2 days/wk 3,5	none
3PD8	3 days/wk 1,2,3	2 days/wk 2,5	2 days/wk 3,6	2 days/wk 2,5	1 day/wk 2
3PD10[b]	3 days/wk 1,2,3	2 days/wk 1,4	5 days/wk excl. 3,7	5 days/wk excl. 3,7	5 days/wk excl. 3,7
5FA4	3 days/wk 4,5,6	none ---	none ---	none ---	none ---

[a] Each rabbit would be exposed to approximately 300 to 450 tsetses between the ears and shaved back, on any one day.

[b] No exposure to the right ear. Exposed to the left ear and back only.

Rabbit	Pair	Age (mo.)	Sex	Previous status
5FA4[c]	1	12	male	exposed both ears and back
5FB1[c]		12	male	naive
3BE5	2	30	female	exposed both ears and back
5FB2		12	female	naive
3PD8[c]	3	30	male	exposed both ears and back
4AJ10[c]		29	male	naive
3PD10[c]	4	30	female	exp. left ear and back only
5FB3[c]		12	female	naive

[c] Rabbits used in fly probing frequency experiment.

effects were rabbit groups (4 naive; 3 previously exposed; 1 rabbit 3PD10, naive to the right ear but previously exposed to the left ear and back) and fly sex.

b) using only fly blood-meal weights from the shaved back. The main effects were rabbit groups (4 naive; 4 previously exposed) and fly sex.

Experiment 2: Fly blood-meal weights obtained from the left (previously exposed) and right (naive) ears of the same rabbit.

If resistance is localized, and providing fly meal weights are a valid and interpretable parameter to use for measuring resistance, then there may be a difference between the fly blood-meals obtained from a previously exposed and a naive ear in the same rabbit.

Four male rabbits from one litter, and a female from a second, were used. The male rabbits (5HB2, 5HB3, 5HB5, 5HB6; age 9 months) had received exposure to their left ear and back twice a week for a period of 4 months prior to this experiment. The female rabbit (3PD10; age 29 months) had received previous exposure to her left ear and back 5 days a week (excluding days 3 and 7) for 3 months prior to this experiment. The right ear of all rabbits had received no previous exposure. Fly blood-meal weights were compared between the left (previously-exposed) and the right (naive) ear, and between the ear previously exposed 5 days a week to those exposed twice a week. Over a period of 15 days, and using a total of 441 flies, approximately 20 fly blood-meal weights were collected for each fly sex, fed on either ear of all 5 rabbits.

Statistical analysis of experiment 2: Correlation coefficients were determined between fly weight and blood-meal weight. Analysis of variance (SPSS) was performed:

a) for fly blood-meal weights obtained between the previously-exposed and naive ears. Three cases were examined using ear, fly sex and rabbit(s) as main effects:

i) using all 5 rabbits

ii) using only the 4 rabbits previously exposed to their left ears and backs twice a week.

iii) using only rabbit 3PD10 (exposed left ear and back 5 days/wk)

b) for fly blood-meal weights obtained from the ears previously exposed twice a week (4 5HB rabbits grouped together) and an ear previously exposed 5 days/wk (rabbit 3PD10). The main effects were fly sex and rabbit groups.

c) for blood-meal weights obtained from the left (naive) ear using the same rabbit groups as in b).

3.3.4.2 Frequency of probings made by tsetses while feeding

Probing counts were used to determine if more effort is required to obtain a blood meal from a particular area of a previously-exposed rabbit than from a naive rabbit. The number of probes is defined as the number of times a tsetse proboscis is inserted and withdrawn, in whole or in part. Three pair of rabbits, all of which had been used in fly blood-meal experiment 1, had flies exposed to their shaved backs and both ears (Table 9). Rabbits 3PD8 and 5FA4 had received exposure to both ears and back, 3PD10 to the left ear and back only (20 flies were exposed to the right ear in fly meal experiment 1 a month earlier) and 5FB1, 4AJ10 and 5HB3, no exposure (except for approximately 20 fly meal weights obtained from the right ear and back during fly meal experiment 1). The rabbits were paired as indicated in Table 9. Two flies at one time were strapped to the selected area of a rabbit for a period of 6 minutes, during which time the probing frequency was observed and recorded. On any one day, the same number of flies were fed upon the same area of each rabbit in one pair. Only unfed, 24 to 48 hour old teneral tsetses were used. Flies were used only once. Those flies which did not feed were designated with a zero. The total number of flies of either sex, placed on one area of each rabbit, varied between 7 and 20 with a mean of 11.7. A total of 421 flies were observed.

Statistical analysis of fly probing counts: ANOVA (SPSS) was performed using:

a) fly probing counts from the left ear and back. The main effects were groups of rabbits (3 exposed - 3PD10, 3PD8, 5FA4; 3 naive - 4AJ10, 5FB1, 5HB3), rabbit area and fly sex.

b) fly probing counts from the right ear only. Rabbit 3PD10 had received no exposure to the right ear and was thus placed with the naive rabbits. The main effects were rabbit groups (2 exposed - 3PD8, 5FA4; 4 naive - as in a) with the additon of 3PD10) and fly sex.

3.4 Results

3.4.1 Rabbit weights and haematological parameters

3.4.1.1 Rabbits exposed 2 to 3 times a week for 8 months

The difference between control (non-exposed or naive) and experimental (exposed 2 to 3 times a week for an 8 month period) rabbits was not significant (Student's t, $P > 0.05$) with respect to red and white cell counts, haematocrit levels, weight changes and whole blood clotting times (Table 10). The mean weight gains for control and experimental rabbits were +30.2 and +4 g respectively, however one exposed rabbit (4BA7) lost 18% (720 g) of its initial body weight over the 8 month period. Without this weight loss, experimental rabbit mean weight gains would have exceeded control rabbit weight gains. The values of each of the parameters measured were within the range of values obtained by previous authors (see review by Scarborough, 1931). The range of all the parameters fluctuated widely.

3.4.1.2 Rabbits exposed 6 days a week for 20 weeks

Over a 20 week period, each of the 4 heavily exposed (exposed to 250 - 500 flies per day, 6 days a week) rabbits were subjected to approximately 40,000 tsetses. Weights of 2 males, 6CA2 and 6AE1, did not change during this period, except for a loss of 330 g by rabbit 6AE1 during the first two weeks of exposure (Fig. 33). The other male, 6AD7 lost 10% (534 g) of initial body weight by the 12th week of exposure and then remained at that level (Fig. 33). The weight of the female rabbit (6AD8) increased, rising twice to peaks during the 7th and 14th weeks of exposure, reaching a maximum weight gain of 8.3% (478 g) initial body weight during the 14th week (Fig. 33). Weights of the 3 control rabbits (receiving only one, 6 day exposure each during the 12th to 15th weeks)

Table 10. Physiological parameters of control rabbits and rabbits exposed to tsetses 2 to 3 times a week over a period of 8 months.

Parameter	Time after first exposure	Control rabbits (no exposure)			Experimental rabbits (exposed)			Student's t
		$\bar{X}$ [a]	S. D.	Range	$\bar{X}$ [a]	S. D.	Range	
Weight (Kg)	5 wk	4.77	0.29	4.34-5.20	4.59	0.15	4.36-4.78	1.20
	32 wk	4.86	0.30	4.51-5.38	4.59	0.33	3.99-5.07	1.24
Red cells ($10^6/\mu l$)	6 wk	8.70	0.97	7.64-10.14	7.65	1.09	6.41-9.14	1.61
White cells ($10^3/\mu l$)	9 wk	8.69	1.32	7.55-10.95	8.06	0.57	7.40-9.05	0.99
Haematocrit (%)	5 wk	39	1.4	37-41.5	38	2.2	35-42	0.91
Coagulation time								
Capillary (sec)	6 wk	270	31	232-335	216	48	120-255	2.10
	9 wk	273	20	240-300	243	41	165-285	1.45
Tube (min)	18 wk	16.25	3.17	12.5-22.5	16.33	4.70	10-23.5	0.03

[a] All means are averages of 6 values. All rabbits are males.

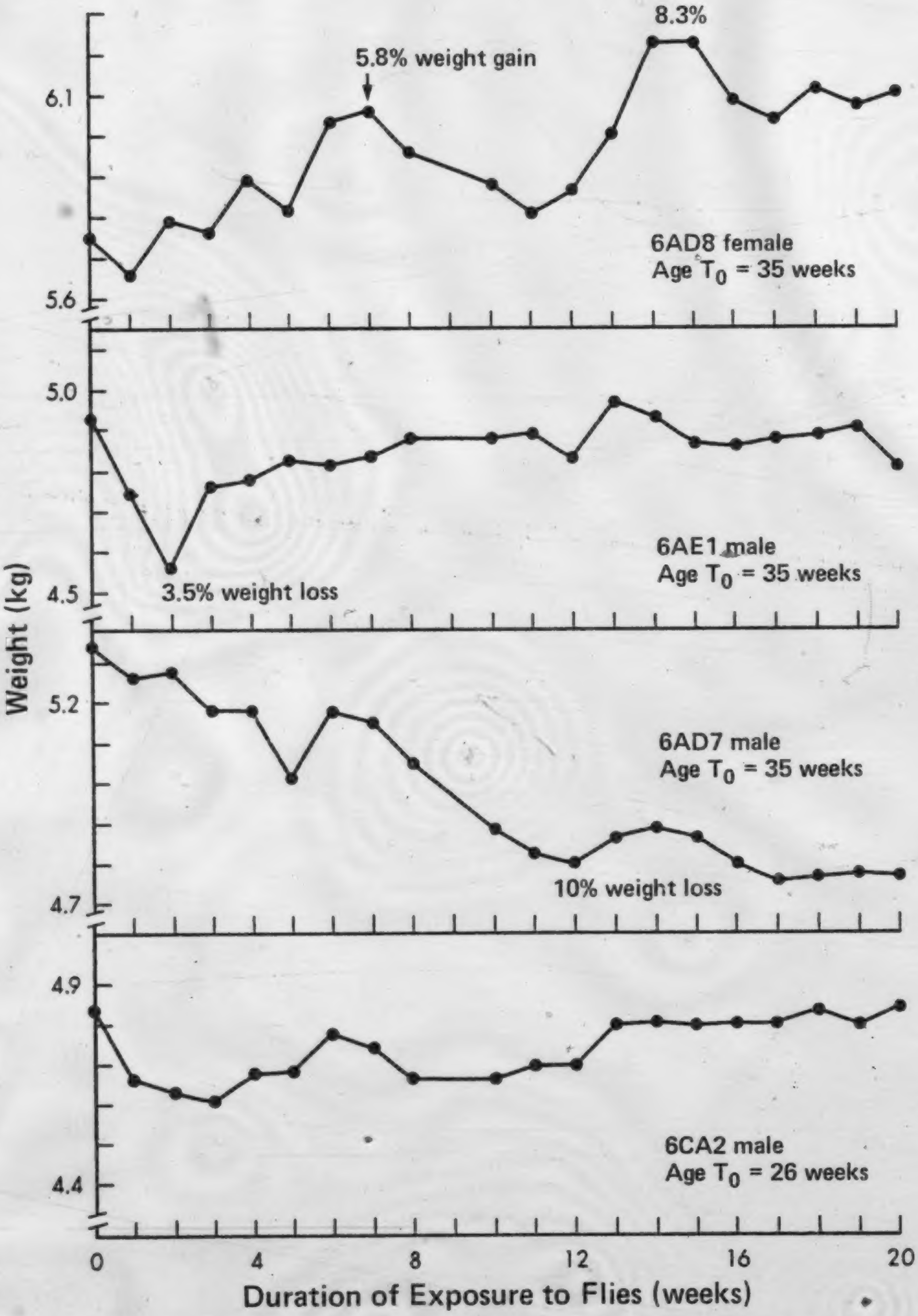


Figure 33. Weights of rabbits exposed 6 days a week for 20 weeks, to exposures of 250-500 flies on each exposure.

increased from weeks 5 to 7 and then decreased slightly (Fig. 34). During the 1 week of tsetse exposure, each of the control rabbits lost 50 to 100 g (Fig. 34), equivalent to 2 to 5 times the estimated weight of blood taken by the tsetses. There was no consistent change in the weights of the control rabbits (Fig. 34).

Haematocrits of all 4 heavily exposed rabbits decreased sharply, from pre-exposure values of 40 to 46% during the 1st to the 3rd weeks, remained stable until the 8th to 10th weeks, and then decreased again (Fig. 35). Haematocrits of each of the 3 control rabbits remained, on average, above 40%, despite weekly bleedings, except for a time period between the 12th and the 15th weeks, when each rabbit was exposed for 6 days (Fig. 36). During this exposure period, haematocrits of each rabbit dropped 2 to 4%.

Daily tsetse exposure for each of the 4 heavily exposed rabbits was recorded. Estimating that 75% of the flies fed daily, and an average meal weight for a tsetse is 0.034 g (Lester and Lloyd, 1928; 0.034 g for male and 0.037 g for female tsetses using G. morsitans), a weekly estimate of the blood loss due to tsetses was obtained (Fig. 35, 37). Cumulative weight loss of blood for each of the 4 heavily exposed rabbits for the entire 20 week period (excluding 10cc removed each week for sera samples) was 1000 g (Fig. 37). This loss is roughly equivalent to 4 times the rabbit's total blood weight (at any one time) estimated from the weight-specific formula 0.055 $B^{0.99}$ (Spector, 1956). The 10cc of blood removed each week for sera samples and citrated plasma was approximately equivalent to the weight of blood in another 175 tsetse blood meals. Over a 20 week period, this blood loss represented another 80% of the rabbit's weight-specific blood weight. Although a correlation existed between weekly blood loss due to tsetses and weekly weight-specific blood weight

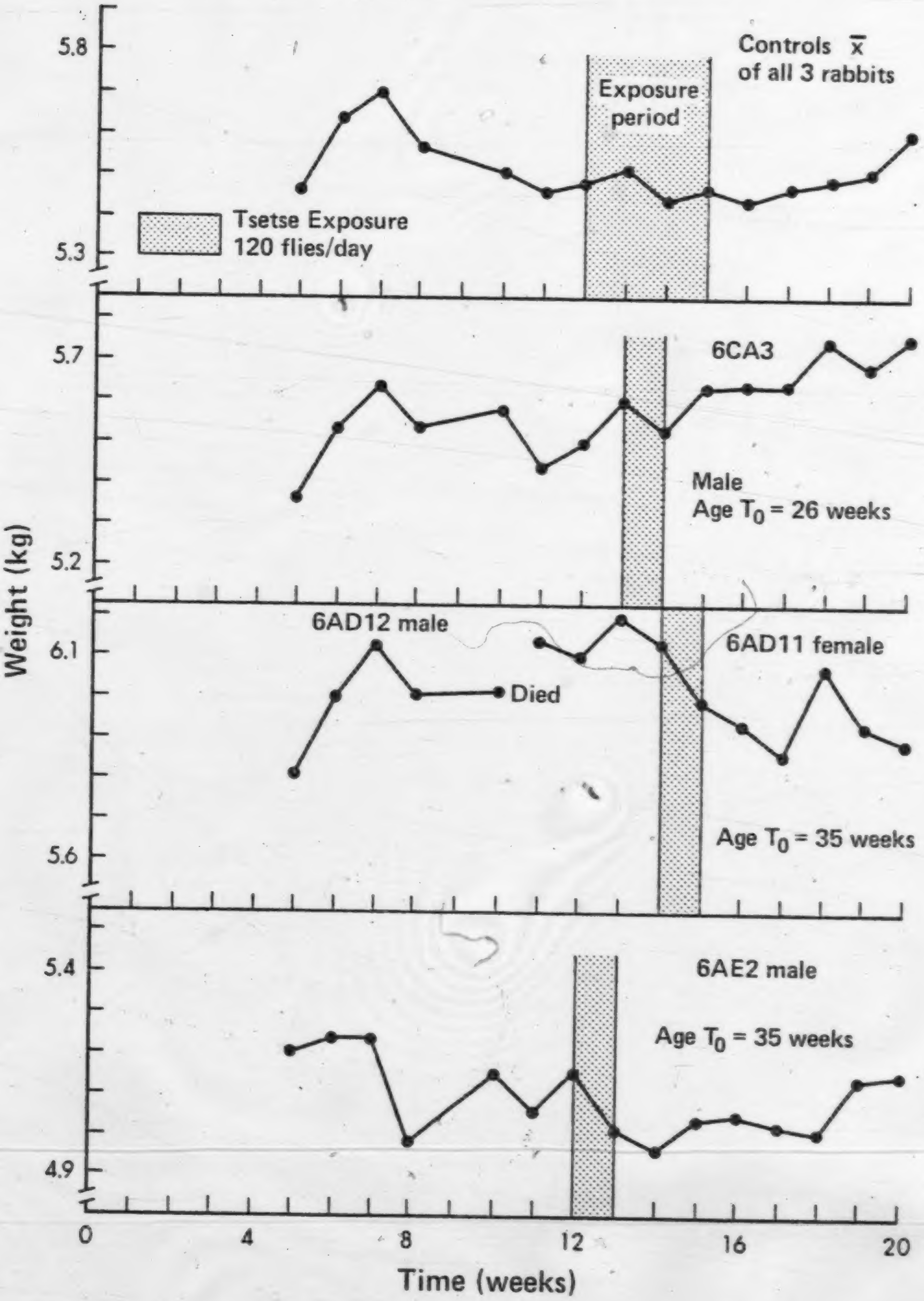


Figure 34. Weights of control rabbits over a 15 week period. Each of the three rabbits received one, 6 day exposure, of 120 flies per day between weeks 12 to 15.

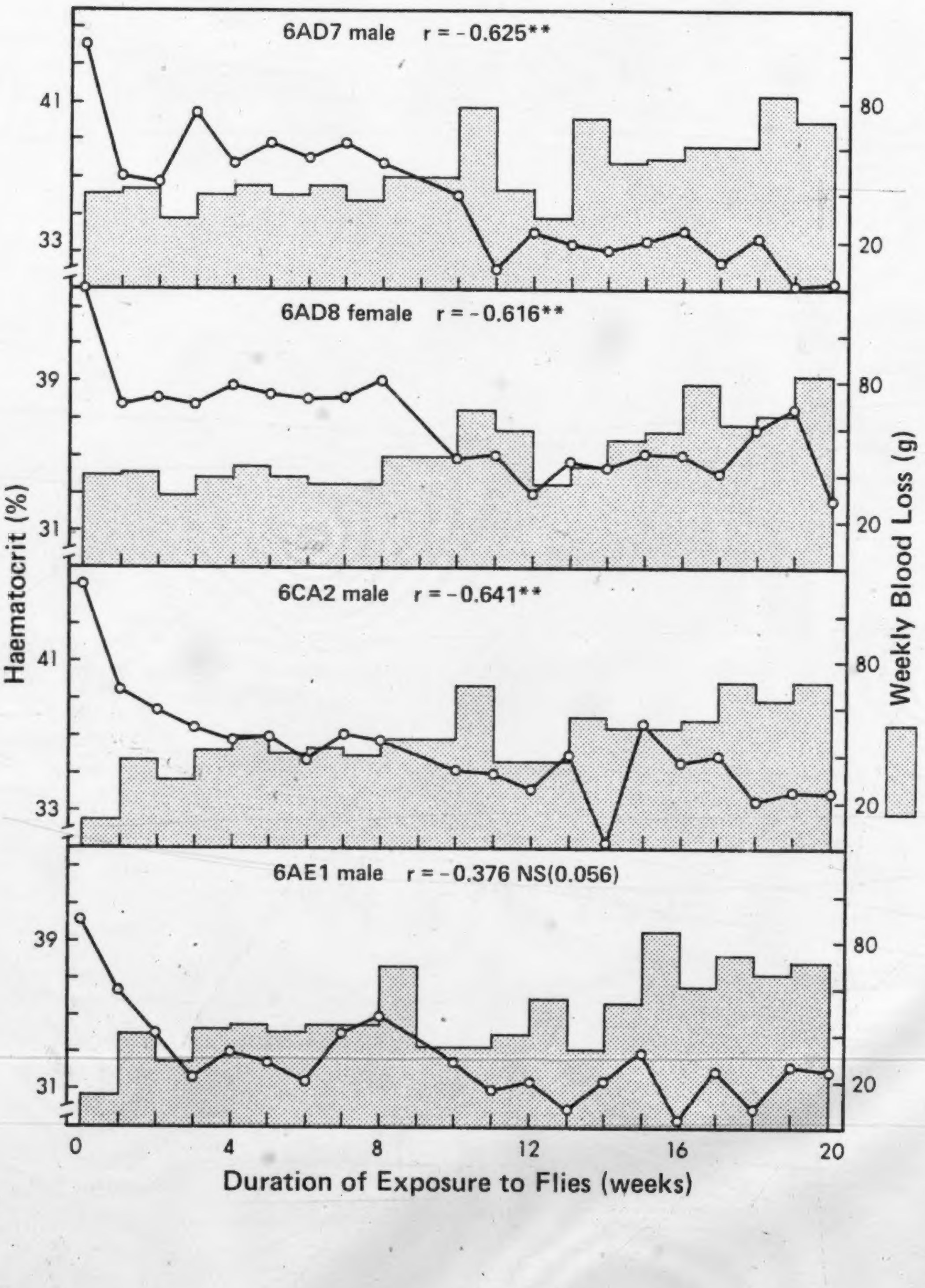
Figure 35. Haematocrits of rabbits exposed to tsetses 6 days a week, for 20 weeks, to 250 - 500 tsetses on each exposure. Shaded area represents the weekly estimated blood loss in the rabbit due to tsetses. There is a negative correlation between the weekly haematocrit values and the estimated weekly blood loss due to tsetses.

r = Spearman correlation coefficients

** = level of significance ($P < 0.01$)

( ) = probability level, short of significance

Correlation coefficients were calculated using weekly estimated blood loss due to tsetses and the haematocrit value at the end of that particular week.


6AD7 male r = -0.625**
6AD8 female r = -0.616**
6CA2 male r = -0.641**
6AE1 male r = -0.376 NS(0.056)
41
33
39
31
41
33
39
31
80
20
80
20
80
20
80
20
Haematocrit (%)
Weekly Blood Loss (g)
0
4
8
12
16
20
Duration of Exposure to Flies (weeks)

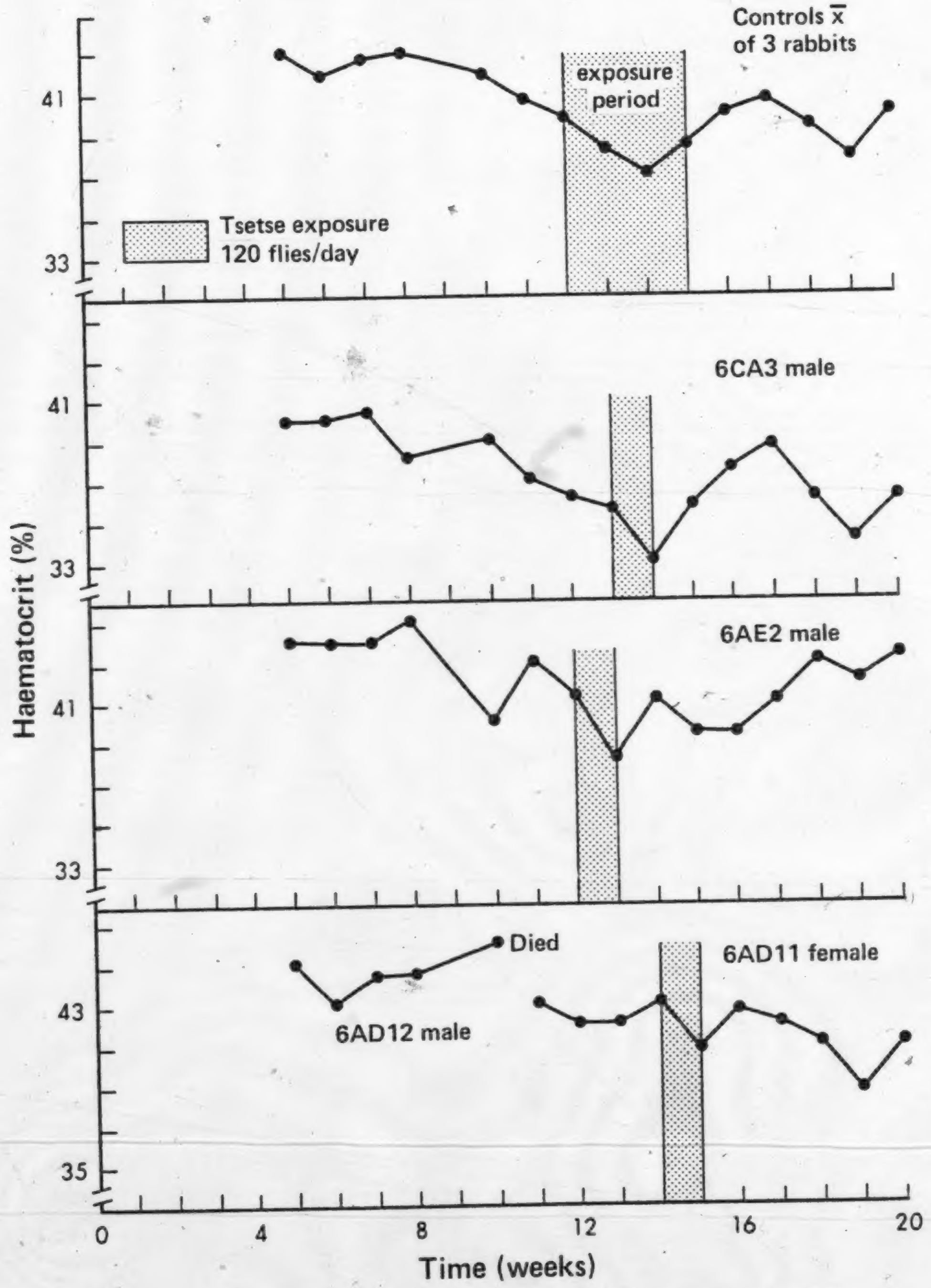


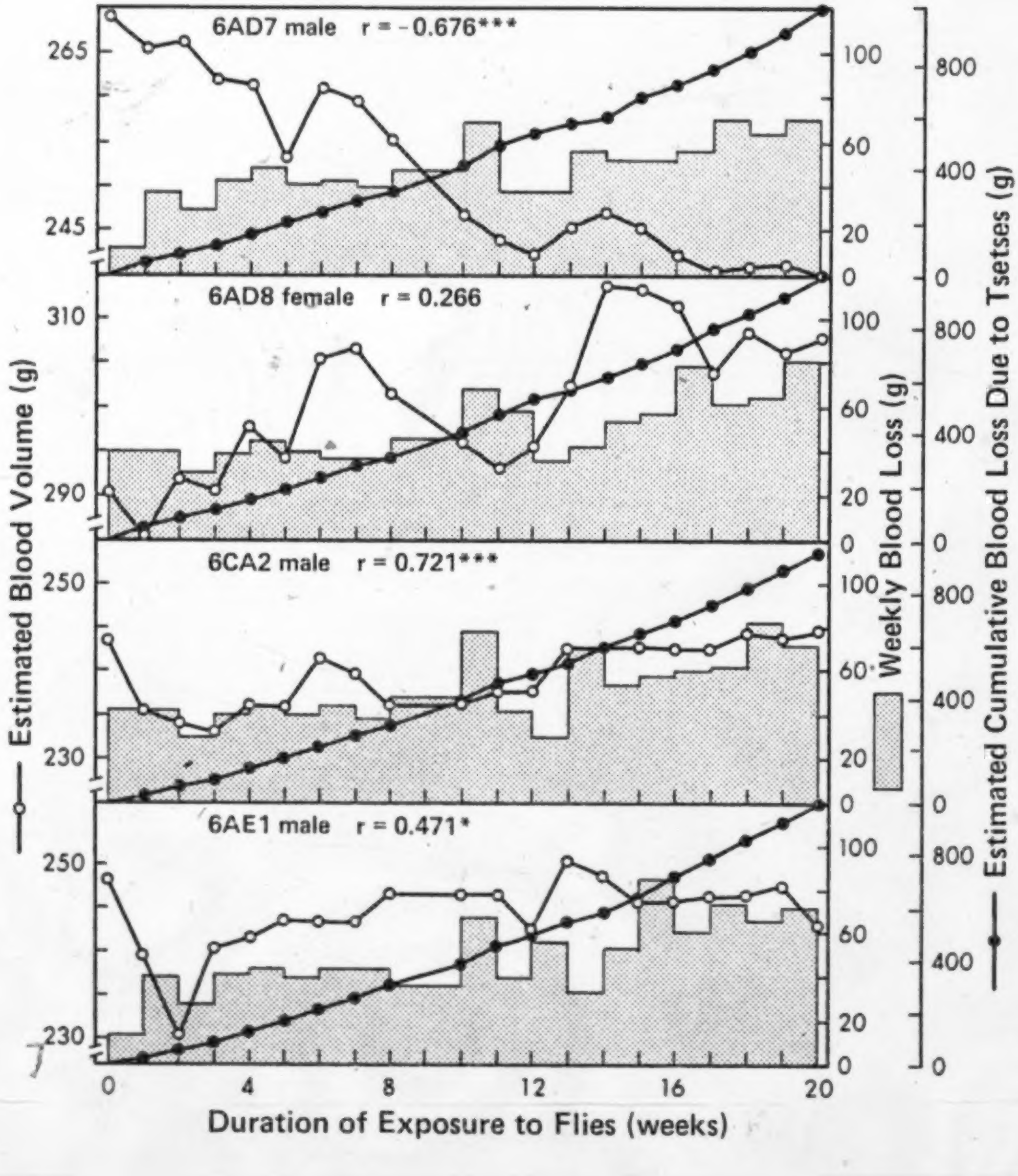
Figure 36. Hematocrits of control rabbits over a 15 week period. During the one week each of the control rabbits received tsetses exposures of 120 flies per day, hematocrits decreased 2 to 4%.

Figure 37. Weekly estimated blood loss due to tsetses, cumulative blood loss due to tsetses, and estimated weight-specific blood weight of rabbits exposed to tsetses 6 days a week, for 20 weeks, to 250 - 500 flies per day. There is no consistent positive or negative correlation between weekly estimated blood loss due to tsetses and weight specific blood weight. Each of the 4 rabbits lost approximately 1000 g of blood during the 20 week exposure period, roughly equivalent to 4 times the weight specific blood weight of each rabbit. The shaded area represents weekly estimated blood loss due to tsetses.

r = Spearman correlation coefficients

Level of significance = * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$

Correlation coefficients were calculated using weekly estimated blood loss due to tsetses and the estimated weight-specific blood weight at the end of that particular week.


6AD7 male r = -0.676***
6AD8 female r = 0.266
6CA2 male r = 0.721***
6AE1 male r = 0.471*
Estimated Blood Volume (g)
Weekly Blood Loss (g)
Estimated Cumulative Blood Loss Due to Tsetses (g)
Duration of Exposure to Flies (weeks)
265
245
310
290
250
230
0
20
60
100
400
800
0
4
8
12
16
20

in 3 of the 4 rabbits (Fig. 37), it was not consistently positive or negative. A significant negative correlation was obtained between weekly blood loss due to tsetses and weekly haematocrit values in each of the 4 heavily exposed rabbits (Fig. 35).

Thrombin times of citrated plasma, taken at weekly intervals from each of the 4 heavily exposed rabbits, fluctuated between 11 and 18 seconds (Fig. 38). Changes in the clotting times during the 8th and 17th weeks were common to all 4 rabbits, indicating a technical artifact rather than a change in host physiology or a change in host physiology affecting all rabbits, but probably independent of their exposure to tsetses. Thrombin times of the 3 control rabbits fluctuated between 11 and 19 seconds with no consistent change in pattern, even during the one week of tsetse exposure (Fig. 39).

3.4.1.3 Rabbits exposed to 1,200 to 1,500 tsetses in 4 hours

Weights of the exposed and control rabbits on the day of exposure or control handling and for a period of 18 to 30 days post-exposure, responded similarly, with the exception of one first time exposed female rabbit, 6DB4. Rabbit 6DB4 gained weight shortly after exposure, reaching a 7.5% (400 g) weight gain by the 9th day post-exposure and an 11% (730 g) increase in weight by the 18th day following exposure (Fig. 40). A female litter mate (6DB5) receiving only control handling, also gained weight, reaching a 2.5% (135 g) increase by the 10th day and a 6% (335 g) increase by the 24th day post-control handling (Fig. 40).

Haematocrits of all 10 rabbits responded similarly (Fig. 41). Control rabbit haematocrits declined 2 to 4% as a result of pre- and post-control handling and bleedings. In the exposed rabbits, receiving, in addition to pre- and post-exposure bleedings, exposure to 1,200 to 1,500 flies

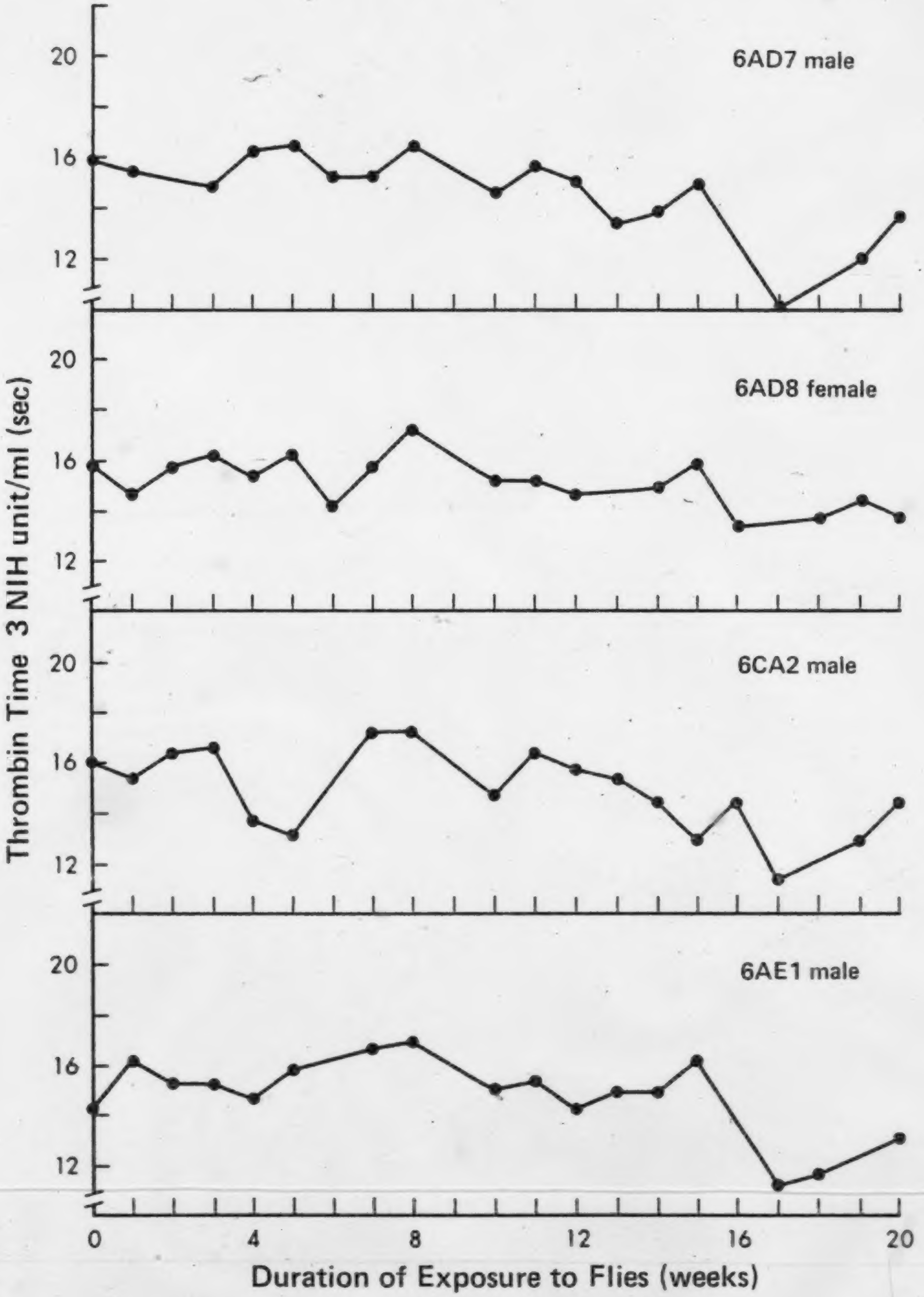


Figure 38. Thrombin times of citrated plasma obtained at weekly intervals from rabbits exposed to tsetses 6 days a week, for 20 weeks, to 250-500 flies on each exposure.

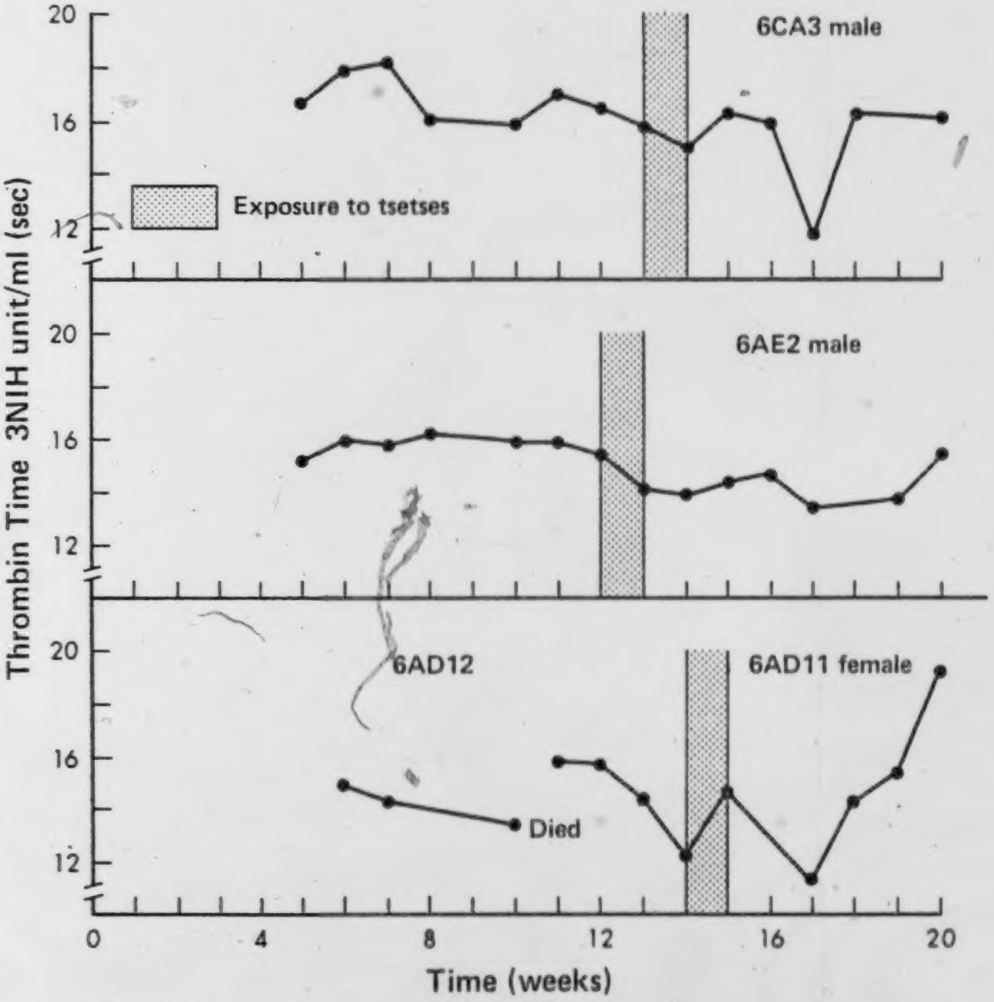


Figure 39. Thrombin times of citrated plasma obtained at weekly intervals from rabbits receiving one weeks exposure of 120 flies per day between the 12th and 15th week.

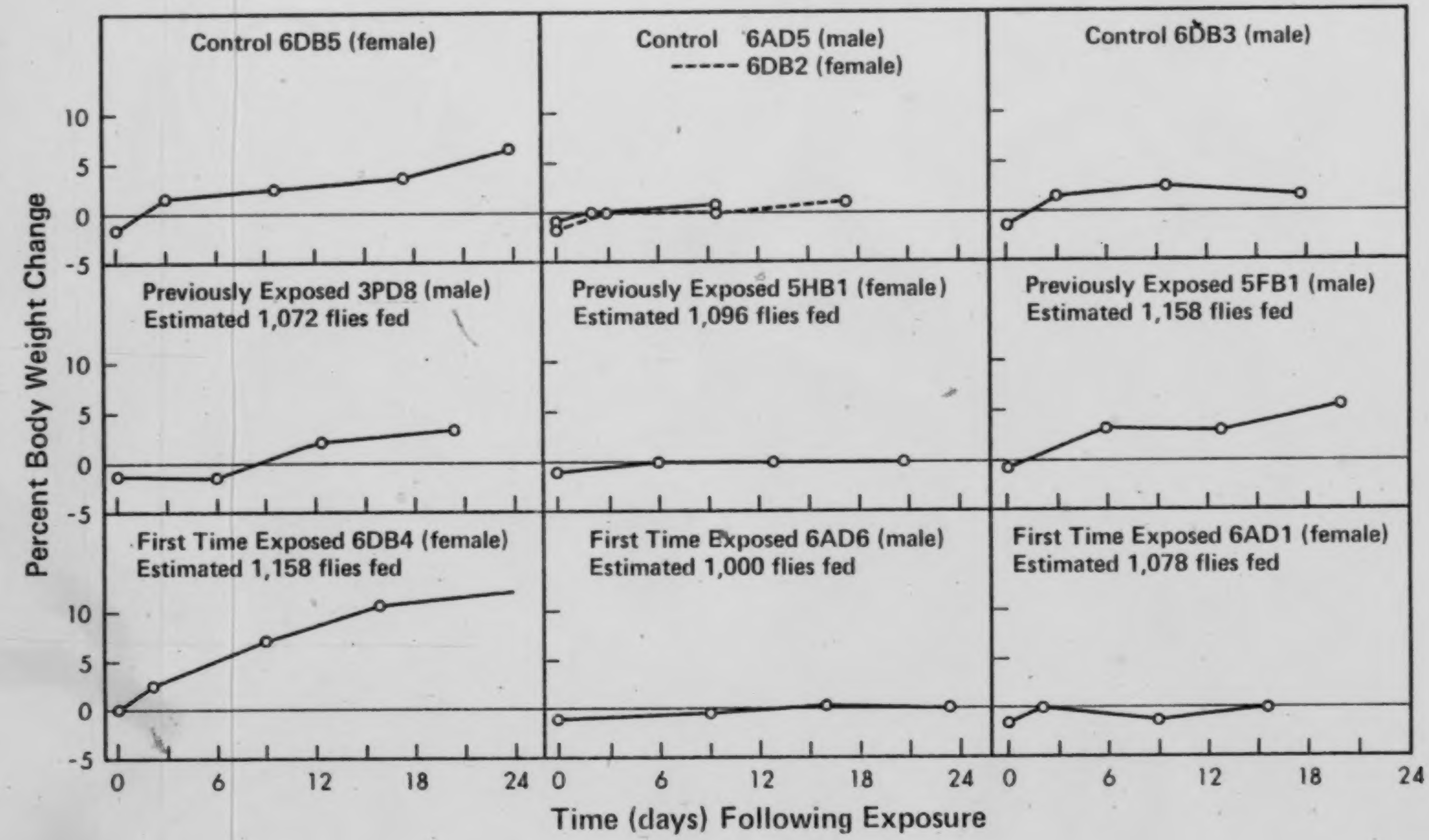


Figure 40. Percent body weight change in rabbits exposed to 1,200 to 1,500 flies within a 4-hour period.

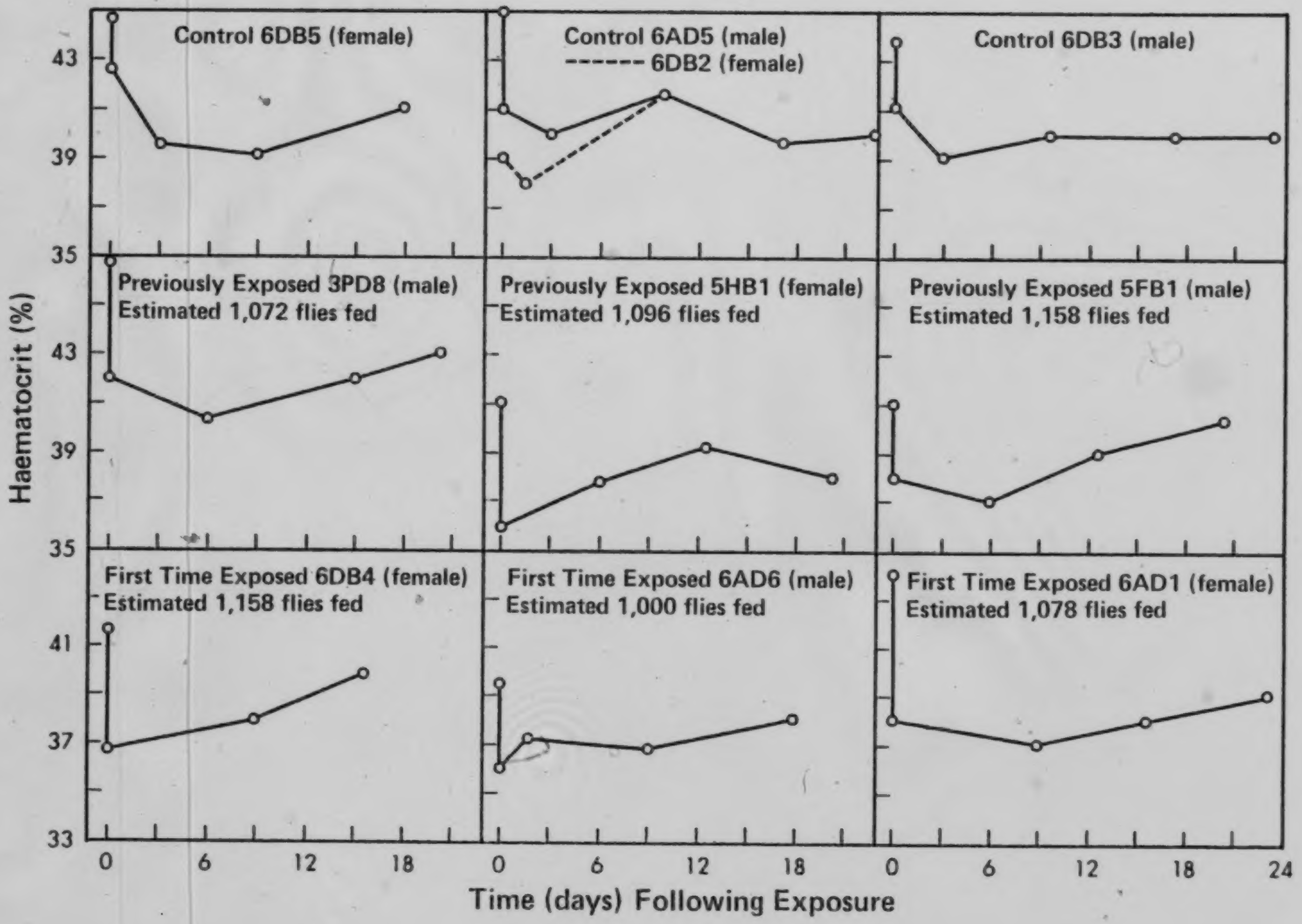


Figure 41. Haematocrits of rabbits exposed to 1,200 to 1,500 flies within a 4-hour period. On day 0, rabbits were bled pre- and post-exposure.

(Fig. 41), haematocrits dropped 3 to 6%. Rabbits heavily exposed to tsetses for the first time responded similar to those rabbits with previous exposure. During the 3 to 4 week post-exposure period, haematocrits of one control, one previously-exposed rabbit, and two rabbits exposed for the first times, returned to pre-exposure levels. Rabbit 6DB4, the rabbit which increased weight following exposure, demonstrated the sharpest decrease in haematocrit (Fig. 41). Haematocrit values of this rabbit returned to normal by the 16th day post-exposure.

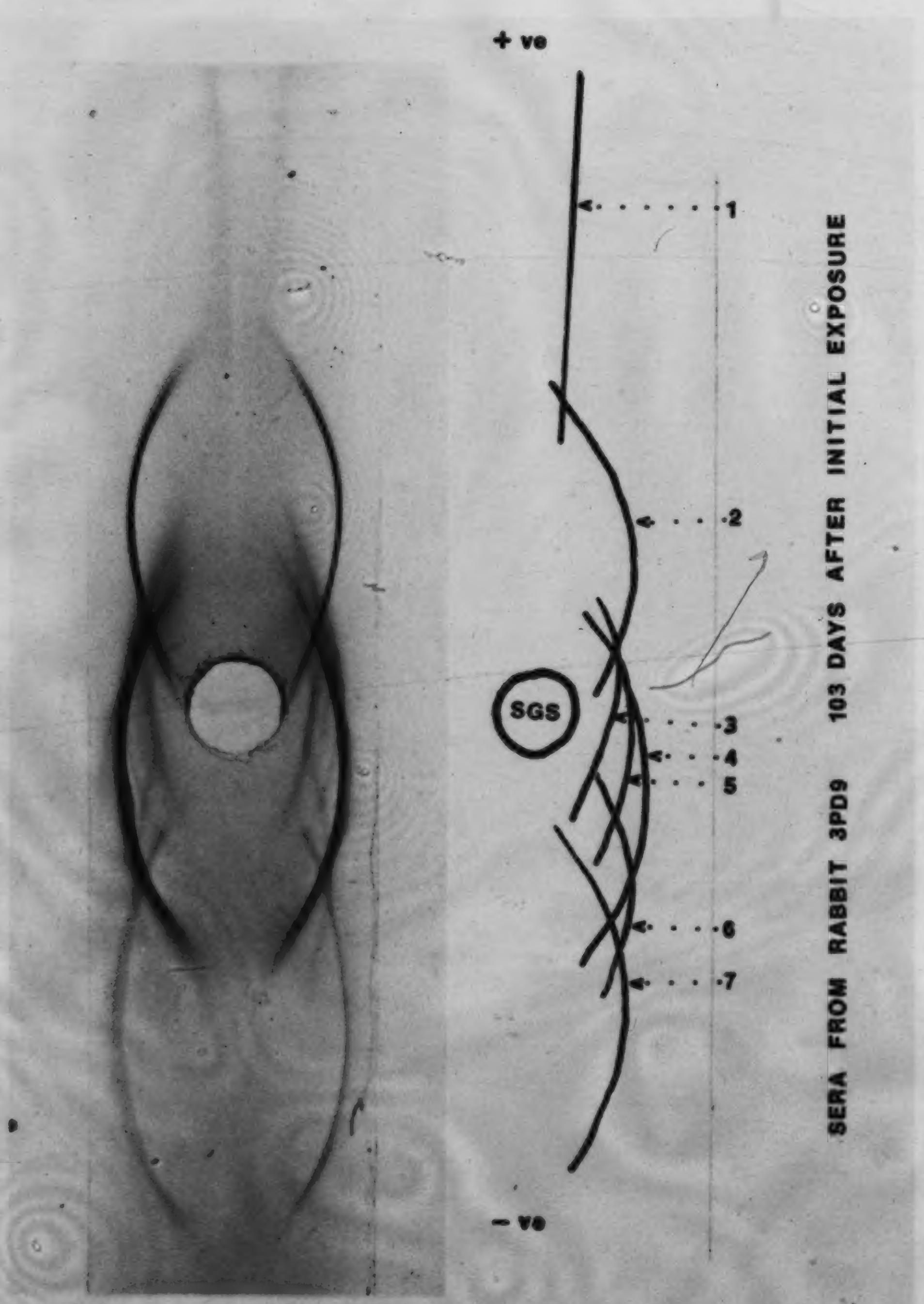
Attempts were made to obtain blood for preparing citrated plasma immediately post-exposure. Bleeding from either exposed ear was extremely difficult, resulting in poor bleedings and clotting of citrated blood. Xylene, placed on the tip of the ear (and later removed) failed to cause dilation of the ear veins. A definite and pronounced arteriolar vasoconstriction occurred in both ears.

3.4.2 Immunological responses to the tsetse

3.4.2.1 Precipitating antibodies

Naturally produced precipitating antibodies against SGS were demonstrated in sera obtained from previously-exposed rabbits using both precipitin ring tests and immunoelectrophoresis (Fig. 42). Positive sera were obtained from all 6 rabbits exposed to tsetses 2 to 3 times a week, all 4 rabbits exposed 6 days a week, and 5 other rabbits receiving various levels of exposure (discussed under passive haemagglutination titre tests). Sera samples from the 3 control rabbits receiving one, 6 day exposure were not examined. Sera samples from control rabbits receiving no exposure, or from experimental rabbits prior to exposure, produced no precipitating antibodies (Fig. 43A).

A maximum of 7 precipitin bands were demonstrated following



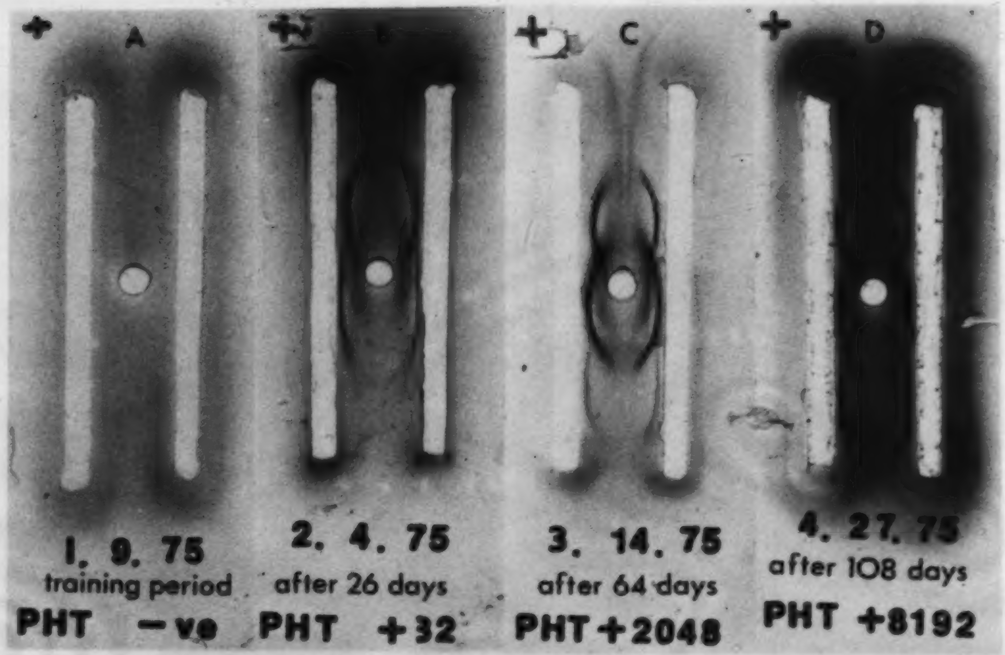
+ ve
1
2
SGS
3
4
5
6
7
– ve
SERA FROM RABBIT 3PD9 103 DAYS AFTER INITIAL EXPOSURE

Figure 42. Immunoelectrophoretic pattern of naturally produced precipitating antibodies of rabbit 3PD9 to tsetse salivary gland solution.

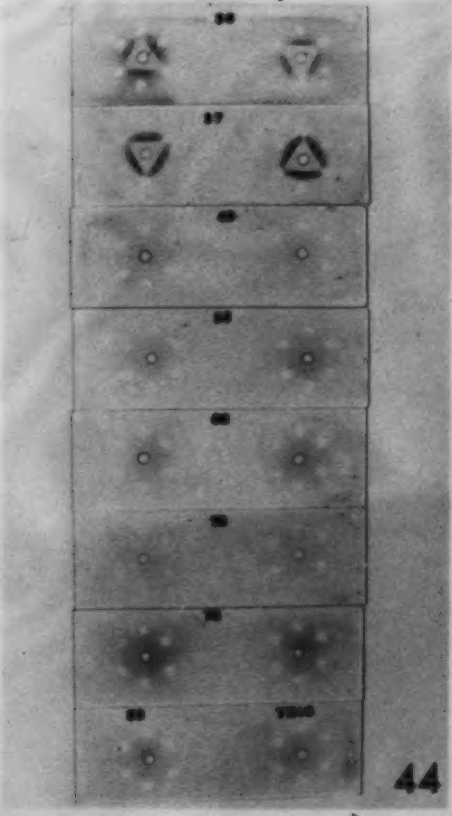
Figure 43. Immunoelectrophoretic pattern of rabbit 3PD5 during the first 108 days following initial exposure. Notice the development of band no. 7 (from Fig. 42). Bands 5 and 6 are not clearly visible. PHT (Passive haemagglutination titres) correspond with immunoelectrophoretic pattern of this rabbit but not for rabbit 3PD9.

Figure 44. Immunodiffusion using Sephadex G-75 fractions of SGS as antigen. Antigenic fractions producing precipitating antibodies with rabbit sera were observed in tube #30 (void volume) to #44. No precipitating antibodies were observed using #53, containing maximum anticoagulant activity.

Figure 45. Example of PHT test using sera of rabbit 4AJ3, 14 weeks after initial exposure. The long row of tubes represent doubling dilutions of rabbit sera mixed with a constant volume of sensitized sheep red cells onto which SGS has been attached. A positive titre of 128 was obtained. In negative tubes, the cells do not agglutinate, but form a button of packed cells on the bottom of the tube. The two tubes on the right are negative controls for non-specific reaction of the antisera and of normal rabbit serum diluent.


+
A
+
+
C
+
D
1. 9. 75
training period
PHT −ve
2. 4. 75
after 26 days
PHT +32
3. 14. 75
after 64 days
PHT +2048
4. 27. 75
after 108 days
PHT +8192

43



44

+128
−ve

45

electrophoresis of SGS and incubation with rabbit 3PD9 sera (Fig. 42). In immunoelectrophoretic patterns of sera samples taken from the same rabbit under a constant level of exposure, more bands formed through a period of time. The first bands formed were usually 2 and 4, followed shortly thereafter by band 7 (Fig. 42, 43). Band 1 was not always observed, even after prolonged exposure, although sera from rabbit 3PD5 indicates its presence on the 26th day following initial exposure (Fig. 43). The last 3 bands observed were no. 3, followed by 5 and 6. Representative example of the changes in the immunoelectrophoretic pattern of rabbit 3PD5, under a constant level of tsetse exposure (6 days a week) is shown in Fig. 43.

Precipitating antibodies were first observed, using the precipitin ring test with rabbit 3PD9 sera, 11 days after initial exposure. During this time, rabbit 3PD9 was exposed on 9 of the 11 days to a cumulative total of 3,331 flies. With the same test, no precipitating antibodies were observed in sera from rabbit 4AJ4 (female) after 2 days exposure to 918 tsetses. However, after 11 days following initial exposure, and being exposed on 10 of those days to a total of 4,610 flies, precipitating antibodies were observed. No sera samples were taken between the 2nd and the 11th day.

There was no difference in the number of precipitin bands observed following immunoelectrophoresis of male and female tsetse salivary glands, although a larger number of male glands were required to produce precipitin patterns similar to those obtained with females. Portions of fed (last fed 12 to 24 hours prior to the experiment) and unfed, male and female tsetse hindguts, anterior and posterior midguts were examined on 2 to 3 occasions for antigenic properties using immunoelectrophoresis. Sera samples which contained precipitating antibodies to SGS did not produce precipitating antibodies with either portion of the tsetse gut.

Sephadex G-75 fractions of SGS were also used as antigen for immunoelectrophoresis and immunodiffusion. Precipitating antibodies were observed substituting fractions #30 (void volume) to #44 in place of SGS (Fig. 44). No precipitating antibodies were observed using selected Sephadex fractions higher than tube #49, including tube fraction #53 which contains maximum anticoagulant activity (Fig. 44). Fractions #45 to #48 were not examined.

3.4.2.2 Passive haemagglutination titre tests (PHT)

Rabbits exposed to tsetses 2 to 3 times a week for 8 months

Circulating antibodies, identified using PHT, were observed in all 4 of the 6 rabbits examined (Fig. 45). Titres of all 4 rabbits increased sharply, the highest titre reaching +1026 by the 55th week following initial exposure in rabbit 3PD6 (Fig. 46a, 46b, 46c, 46d). Titres of sera obtained from rabbits 3PD6 (Fig. 46c) and 3PD7 (Fig. 46a) leveled off at +256 and remained stable. Titres of rabbits 4AJ3 (Fig. 46d) and 4AJ2 (Fig. 46b) decreased during a period of constant tsetse exposure. Titres of rabbit 4AJ2 (Fig. 46b) returned to zero following 20 weeks of no exposure.

Rabbits exposed 6 days a week for 20 weeks

Using PHT, circulating antibodies were identified in all 3 of the 4 rabbits exposed 6 days a week for 20 weeks (time did not permit examination of the 4th rabbit). Titres in the male rabbits 6AD7 and 6CA2, were first detected at the end of the second week of exposure, and then increased slowly throughout the 20 week period (Fig. 47a, 47b). Titres of the male rabbits were considerably lower than those obtained

Figure 46. Passive haemagglutination titres of 4 rabbits exposed to 250 - 500 tsetses per day, 2 to 3 days per week, for a period of at least 10 months. Titres of all 4 rabbits increased sharply from 0 following initial exposure. Titres of rabbits 4AJ2 and 4AJ3 decreased during a period of constant exposure, while titres of rabbits 3PD6 and 3PD7 remained relatively constant.

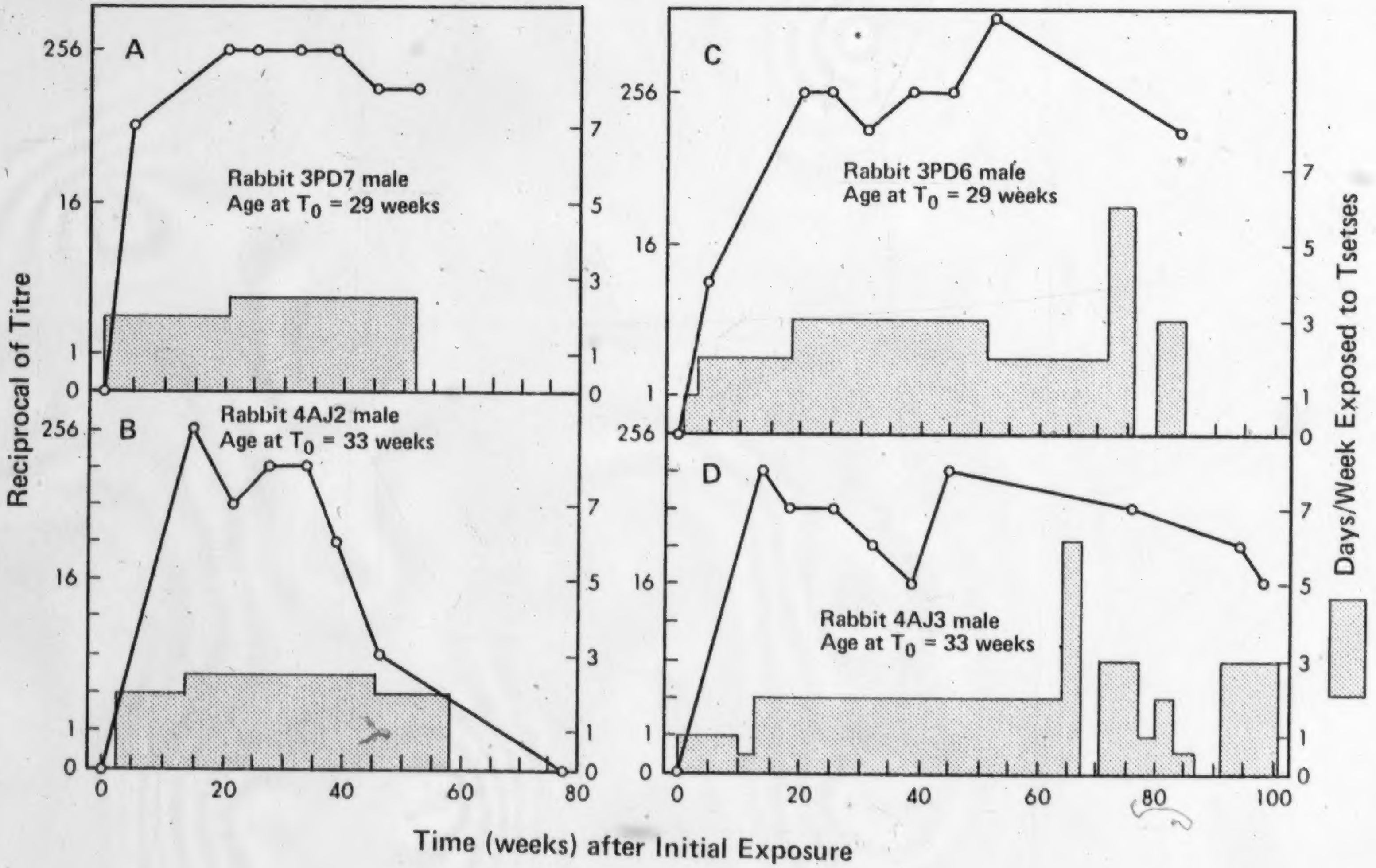
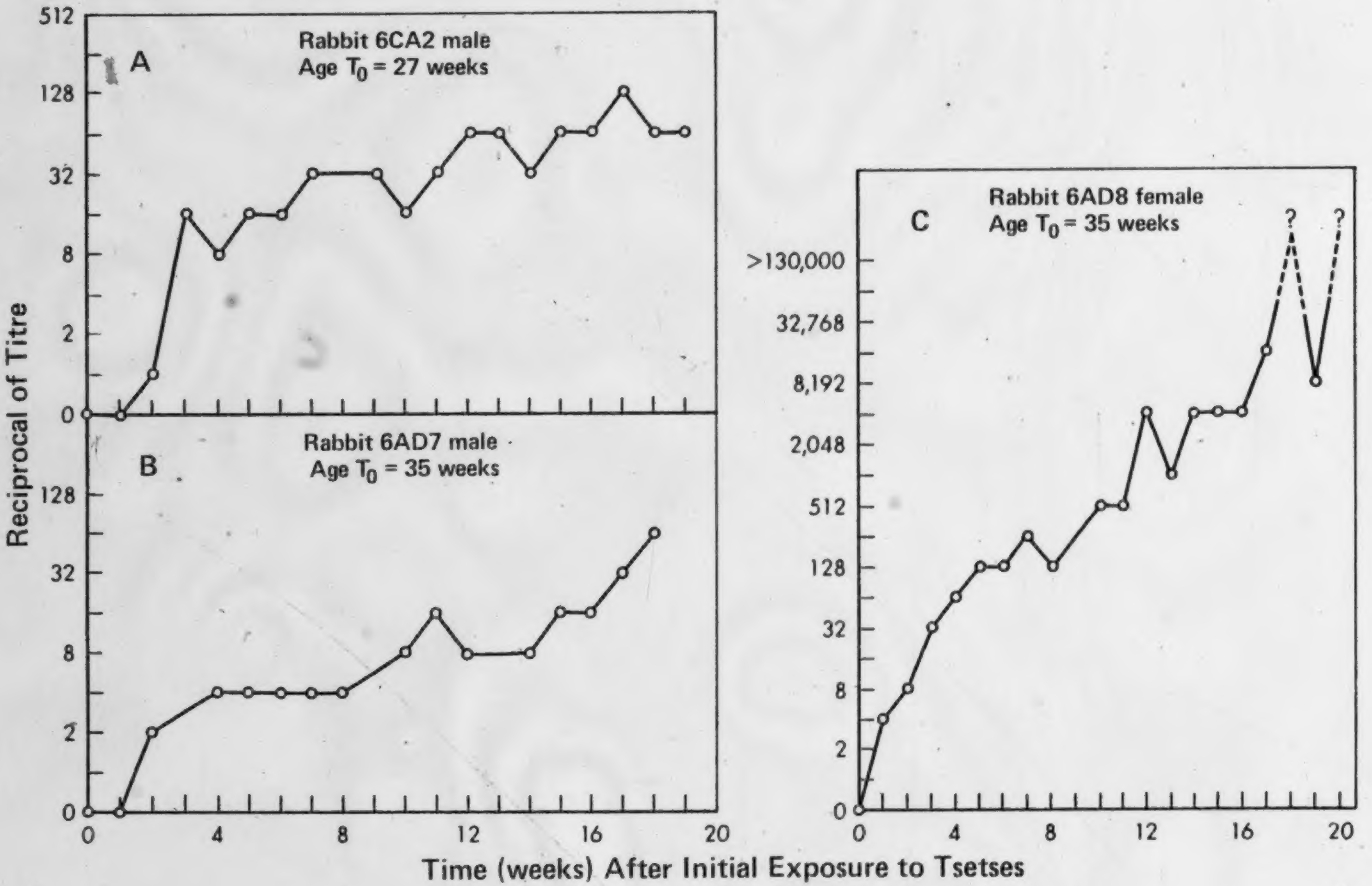

A
Rabbit 3PD7 male
Age at T_0 = 29 weeks
B
Rabbit 4AJ2 male
Age at T_0 = 33 weeks
C
Rabbit 3PD6 male
Age at T_0 = 29 weeks
D
Rabbit 4AJ3 male
Age at T_0 = 33 weeks
Reciprocal of Titre
256
16
1
0
Days/Week Exposed to Tsetses
7
5
3
1
0
20
40
60
80
100
Time (weeks) after Initial Exposure

Figure 47. Passive haemagglutination titres of 3 rabbits exposed to 250 - 500 flies per day, 6 days a week, for 20 weeks. Titres of the two male rabbits (A, B) rose very slowly throughout the 20 week period, with the first positive sera being obtained at the end of the second week of exposure. In contrast, titres of the female rabbit (C) rose sharply and steadily throughout most of the 20 week period. Positive titres were first observed after the first week of exposure.


Reciprocal of Titre
A
Rabbit 6CA2 male
Age T_0 = 27 weeks
B
Rabbit 6AD7 male
Age T_0 = 35 weeks
C
Rabbit 6AD8 female
Age T_0 = 35 weeks
?
?
512
128
32
8
2
0
>130,000
32,768
8,192
2,048
0
4
8
12
16
20
Time (weeks) After Initial Exposure to Tsetses

from the female rabbit, 6AD8 (Fig. 47c). In rabbit 6AD8, titres were first detected after one week of exposure, and continued to rise throughout the 20 week period with the 18th and 20th weeks reaching beyond +130,000 (Fig. 47c).

Titre response during the second period of exposure

For 4 rabbits, 3PD1, 3PD5 3PD9 (males) and 3PD4 (female) titres were determined during an initial exposure of 6 days/week, followed by a period of no exposure and then during a second period of exposure (variable exposure; Fig. 48a, 48b, 48c, 48d). During the initial period of exposure, titres of rabbits 3PD4 and 3PD9 rose sharply to +512 and +32,768 respectively (Fig. 48c, 48d). While under a constant level of exposure, titres of rabbit 3PD9 declined by the 6th week to +4096, and then still further to +512 during the 15th week of exposure (Fig. 48d). With a constant level of tsetse exposure, titres from the sera of rabbits 3PD1 and 3PD5 increased more slowly than the other two rabbits, to +1024 and +8192 respectively, and then leveled off (Fig. 48a, 48b). After the initial exposure period, each of these rabbits received no exposure for a period lasting 4 to 20 weeks, During this time, titres of rabbits 3PD1, 3PD4 and 3PD5 decreased (Fig. 48a, 48b, 48c) although remaining within 4 doubling dilutions of titres obtained during the first level of exposure. Sera samples from rabbit 3PD9 were not obtained immediately following its 4 week rest period. With a second period of exposure, titres of rabbits 3PD1 (Fig. 48a) and 3PD5 (Fig. 48b) increased higher than titres obtained during the first level of exposure, to a point outside of the range of titres examined. In contrast, titres of rabbits 3PD4 remained stable during the second phase of exposure (Fig. 48c) while titres of 3PD9 declined (Fig. 48d).

Figure 48. Passive haemagglutination titres of rabbits receiving intermittent tsetse exposure.

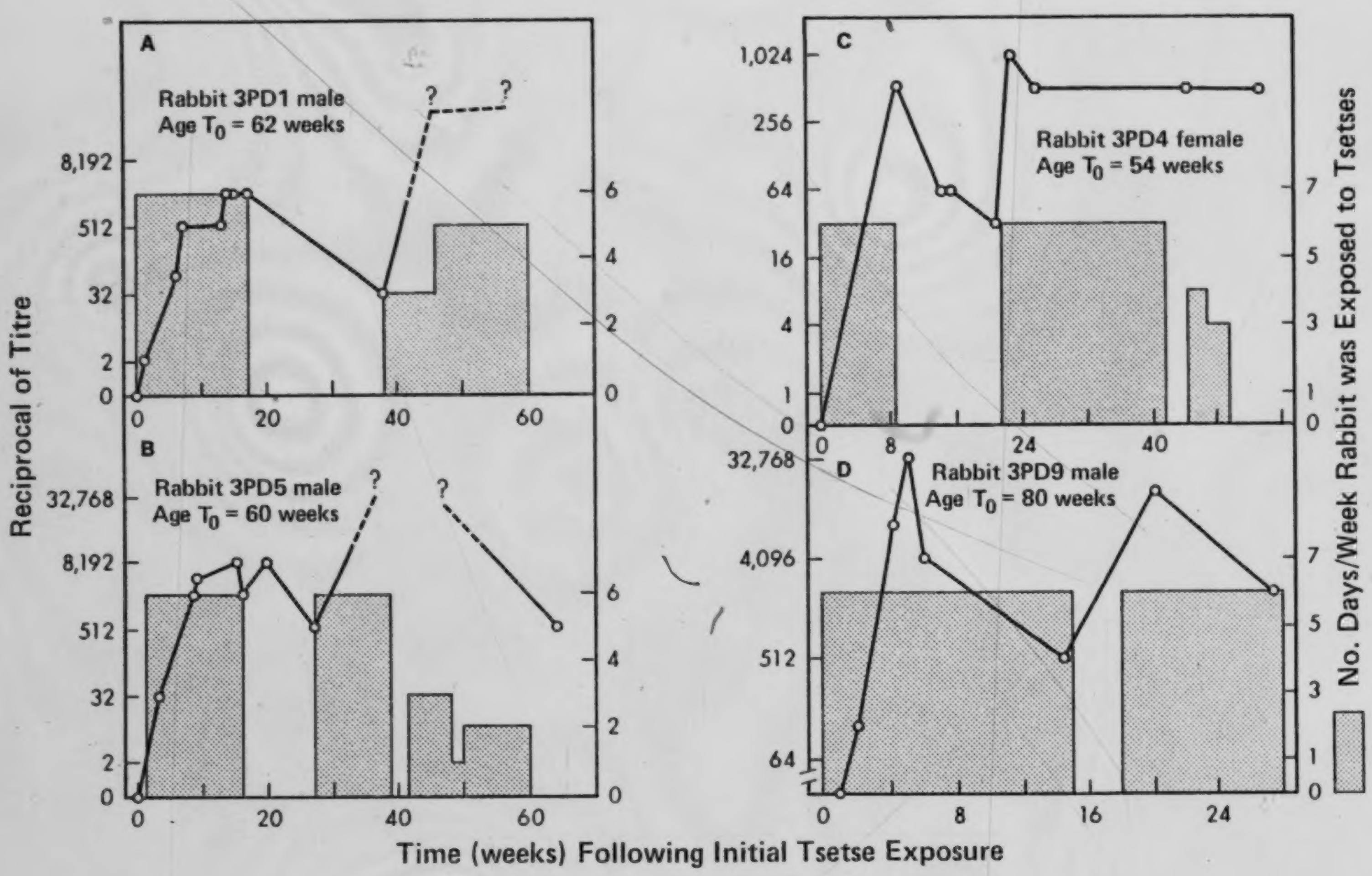
A) Rabbit 3PD1 received exposure for a 17 week period, followed by a period of no exposure (20 weeks) and then was re-exposed.

B) Rabbit 3PD5 received exposure for a 16 week period, followed by a period of no exposure (10 weeks) and then was re-exposed.

C) Rabbit 3PD4 received exposure for the first 8 weeks, rested for 13 weeks and then received exposure again during the 21st week.

D) Rabbit 3PD9 received exposure for the first 15 weeks, rested for 4 weeks and then was re-exposed again.

Titres correlate, in part, with tsetse exposure.


A
Rabbit 3PD1 male
Age T_0 = 62 weeks
?
?
8,192
512
32
2
0
0
20
40
60
6
4
2
0
B
Rabbit 3PD5 male
Age T_0 = 60 weeks
?
?
32,768
8,192
512
32
2
0
0
20
40
60
6
4
2
0
Reciprocal of Titre
C
Rabbit 3PD4 female
Age T_0 = 54 weeks
1,024
256
64
16
4
1
0
0
8
24
40
7
5
3
1
0
D
Rabbit 3PD9 male
Age T_0 = 80 weeks
32,768
4,096
512
64
0
8
16
24
7
5
3
1
0
No. Days/Week Rabbit was Exposed to Tsetses
Time (weeks) Following Initial Tsetse Exposure

In another male rabbit, 3PD8, titres were examined during a short, low level of exposure, and then to a high level of exposure after 46 weeks of no exposure (Fig. 49). Titres were low during the initial short, low level of exposure, and returned to zero 16 weeks following the initial exposure period. At week 54, after 46 weeks of no exposure, this rabbit was exposed to tsetses 6 days a week. Although titres did not rise for two and a half weeks, within 12 weeks, titres reached levels of +130,000. Titres remained above this level for at least another 12 weeks, and then after 42 weeks of exposure to tsetses 6 days a week, and 95 weeks after the start of the experiment, titres dropped to +32,768 (Fig. 49).

Correlation of PHT with immunoelectrophoretic patterns

In rabbit 3PD5, PHT titres increased as the number of immunoelectrophoretic bands increased (Fig. 43). With the immunoelectrophoretic pattern obtained from rabbit 3PD9 (not shown), similar to the pattern of 3PD5, titres increased at 14 (+128), 28 (+8192) and 35 days (+32,768), but decreased at 103 days (+512).

3.4.3 Tests for skin resistance

3.4.3.1 Fly blood-meal weights

Experiment 1: Fly meal weights from the right ear and back of previously-exposed and naive rabbits.

Meal weights of flies feeding on the right ear or the back of previously-exposed rabbits were not significantly different from those feeding on naive rabbits (Tables 11, 12; Appendix C, Table C1). Meal weights taken by female flies were significantly heavier than those taken by males (Tables 11, 12; Appendix C, Table C1). Fly meal weights taken from the naive, right ear of rabbit 3PD10 (a rabbit whose left ear and back had been exposed to tsetses) were on average 1.55 mg lower than meal

Figure 49. Passive haemagglutination titres of rabbit 3PD8. After a short low level of exposure, corresponding with low PHT, titres returned to 0, 16 weeks following initial exposure. A heavy exposure period, 46 weeks after the initial exposure, resulted in titres over +130,000.

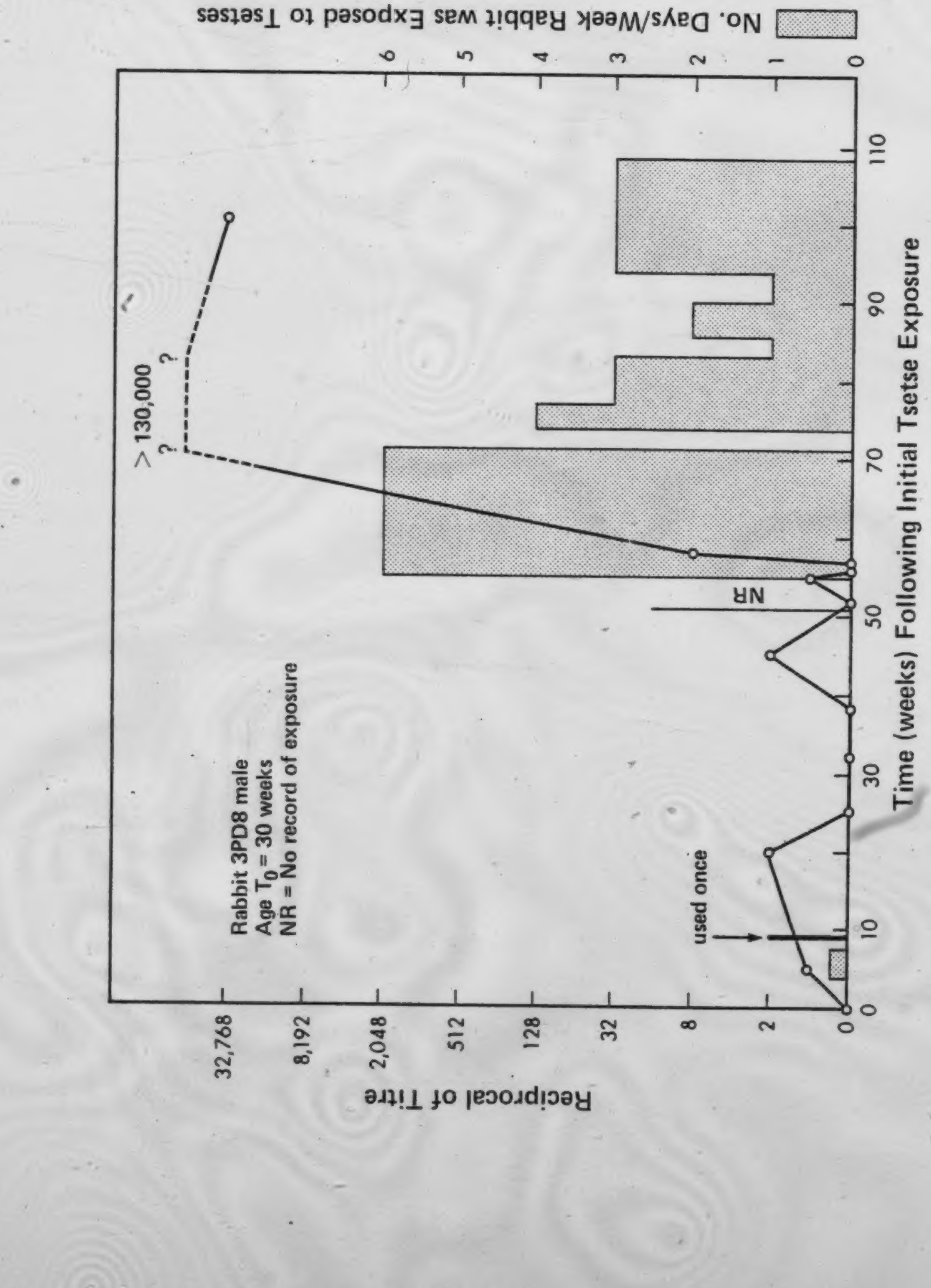

Rabbit 3PD8 male
Age T_0 = 30 weeks
NR = No record of exposure
used once
NR
> 130,000
?
?
Reciprocal of Titre
0
2
8
32
128
512
2,048
8,192
32,768
Time (weeks) Following Initial Tsetse Exposure
0
10
30
50
70
90
110
0
1
2
3
4
5
6
▨ No. Days/Week Rabbit was Exposed to Tsetses

Table 11. ANOVA of fly blood-meal weights from the right ear of previously-exposed and naive rabbits

Source of variation	Sum of squares	DF	Mean square	F
Rabbits	103.24	2	51.62	0.931
Fly sex	1534.40	1	1534.40	27.68 ***
Interaction:				
Rabbit Fly sex	153.07	2	76.53	1.38
Residual	12695.18	229	55.43	
Total	14462.98	234	61.80	

Multiple classification analysis Grand mean = 25.45 mg

Variable and category	N	Deviation from mean (mg)
Rabbit group:		
Non-exposed rabbits (4)[a]	118	0.09
Exposed rabbits (3)[b]	89	0.36
No exposure 3PD10[c]	28	- 1.55
Fly sex:		
Female	117	2.55
Male	118	- 2.53

Level of significance *** $P < 0.001$

[a] Non-exposed rabbits = 5FB1, 5FB2, 5FB3, 4AJ10

[b] Previously-exposed rabbits = 3BE5, 3PD8, 5FA4

[c] Exposed to the left ear and back, but never the right ear

( ) = no. of rabbits

Table 12. ANOVA of fly blood-meal weights from the back of previously-exposed and naive rabbits

Source of variation	Sum of squares	DF	Mean square	F
Rabbits	13.51	1	13.51	0.141
Fly sex	667.37	1	667.37	6.990 ***
Interaction:				
Rabbit Fly sex	8.13	1	8.13	0.085
Residual	24254.21	254	95.49	
Total	24943.43	257	97.06	

Multiple classification analysis Grand mean = 23.33 mg

Variable and category	N	Deviation from mean (mg)
Rabbit group:		
Non-exposed rabbits (4)[a]	130	- 0.23
Previously-exposed rabbits (4)[b]	128	0.23
Fly sex:		
Female	147	1.40
Male	111	- 1.85

Level of significance *** $P < 0.001$

[a] Non-exposed rabbits = 5FB1, 5FB2, 5FB3, 4AJ10

[b] Previously-exposed rabbits = 5FA4, 3PD8, 3PD10, 3BE5

( ) = no. of rabbits

weights of flies taken from the previously-exposed or naive, right ears of other rabbits. However, this difference was not significant (Table 11). The mean meal weight taken by flies from the right ear (25.45 mg) was significantly larger (Student's t, $P < 0.05$) than the meal weight taken from the back (23.33), indicating that regardless of whether the back has been previously exposed or not, it is more difficult for flies to feed there.

Experiment 2: Fly meal weights obtained from the left (previously exposed) and right (naive) ears of rabbits.

There was no significant difference ($P > 0.05$) in the fly meal weights between the previously exposed and naive ears, regardless of whether the previously exposed ear had been exposed twice or 5 times a week (Table 13, 14, 15; Appendix C, Table C2). However, flies took lower meal weights from rabbit 3PD10 (exposed to the left ear and back 5 days/week) than from the other 4 rabbits (exposed to the left ear and back twice/week) (Table 13). There were no differences in the fly meal weights between the 4 rabbits previously exposed twice a week to their left ears and backs (Table 15). Fly meal weights, regardless of whether obtained from the previously-exposed, left ear or the naive, right ear, were significantly larger from the 4 rabbits previously exposed to their left ear and back twice a week, than from rabbit 3PD10, previously exposed to the left ear and back 5 days/week (Table 16, 17; Appendix C, Table C2). Meal weights obtained by female flies were significantly ($P < 0.05$) larger than meal weights taken by male flies (Table 13, 14, 15, 16; Appendix C, Table C2). Fly blood-meal weights correlated with fly weights in 9 of 20 cases (Table 18). There was no tendency for a significant correlation between fly weight and meal weight to be related to fly sex or to source of the

Table 13. ANOVA of fly blood-meal weights from the left (previously-exposed) and right (naive) ears of rabbits.

Source of variation	Sum of squares	DF	Mean square	F
Rabbits	824.8	4	206.2	4.04 **
Fly sex	4375.6	1	4375.6	85.73 ***
Ear	8.2	1	8.2	0.16
2 way interaction:				
Rabbit Fly sex	85.0	4	21.3	0.42
Rabbit Ear	66.6	4	16.6	0.33
Fly sex Ear	17.3	1	17.3	0.34
3 way interaction:				
Rabbit Fly sex Ear	17.1	4	17.3	0.08
Residual	21486.8	421	51.0	
Total	26822.5	440	60.9	

** $P < 0.01$ *** $P < 0.01$

Multiple classification analysis

Grand mean = 24.98 mg

Variable and category	Status	N	Deviation from mean (mg)
Rabbit:			
5HB2	left ear and back exposed 2 days/wk	108	1.40
5HB3		82	0.06
5HB5		79	- 0.21
5HB6		79	0.93
3PD10	left ear and back exp. 5 days/wk	93	- 2.29
Fly sex:			
Female		246	2.78
Male		195	- 3.51
Ear:			
Left	exposed	225	- 0.15
Right	naive	216	0.15

Table 14. ANOVA of fly blood-meal weights taken from the previously-exposed (left) and the naive (right) ear of rabbit 3PD10.

Source of variation	Sum of squares	DF	Mean square	F
Fly sex	654.3	1	654.3	8.48 **
Ear	42.1	1	42.1	0.55
Interaction:				
Fly sex Ear	35.0	1	35.0	0.45
Residual	6942.7	90	77.1	
Total	7691.3	93	82.7	

Multiple classification analysis Grand mean = 22.48 mg

Variable and category	Status	N	Deviation from mean (mg)
Fly sex:			
Female		57	2.15
Male		37	- 3.32
Ear:			
Left	previously exposed	51	0.73
Right	naive	43	- 0.87

Level of significance ** $P < 0.01$

Table 15. ANOVA of fly blood-meal weights taken from the previously-exposed and naive ears of 4 rabbits previously exposed twice a week to 1 ear

Source of variation	Sum of squares	DF	Mean square	F
Rabbit	51.8	3	17.29	0.383
Fly sex	3613.5	1	3613.5	79.997 ***
Ear	26.5	1	26.5	0.588
2 way interaction:				
Rabbit Fly sex	76.1	3	25.4	0.562
Rabbit Ear	34.3	3	11.4	0.253
Fly sex Ear	7.4	1	7.4	0.165
3 way interaction:				
Rabbit Fly sex Ear	12.4	3	4.1	0.091
Residual	14996.6	332	45.2	
Total	18919.7	347	54.5	

Multiple classification analysis Grand mean = 25.59

Variable and category	Status	N	Deviation from mean (mg)
Rabbit:			
5HB2	previously exposed to the left ear and back 2 days/wk., no exposure to the right ear	108	0.79
5HB3		82	- 0.55
5HB5		79	- 0.82
5HB6		79	0.32
Fly sex:			
Female		190	2.98
Male		158	- 3.58
Ear:			
Left	previously exposed	174	- 0.28
Right	naive	174	0.28

Level of significance *** $P < 0.001$

Table 16. ANOVA of fly blood-meal weights of teneral tsetses fed on the naive, right ear of rabbits exposed twice or 5 times/week to their left ears and backs

Source of variation	Sum of squares	DF	Mean square	F
Rabbits	688.2	1	688.2	14.1 ***
Fly sex	1817.1	1	1817.2	37.4 ***
2 way interaction:				
Rabbit Fly sex	39.9	1	39.9	0.8
Residual	10334.7	213	48.5	
Total	12818.4	216	59.3	

Multiple classification analysis Grand mean = 25.03 mg

Variable and category	Status	N	Deviation from mean (mg)
Rabbit group:			
4 rabbits[a]	exposed 2 days/wk on the left ear and back. No exposure to right ear.	174	0.84
1 rabbit - 3PD10	exposed 5 days/wk to the left ear and back. No exposure to right ear.	43	- 3.42
Fly sex:			
Female		120	2.56
Male		97	- 3.16

[a] Rabbits 5HB2, 5HB3, 5HB5, 5HB6

Level of significance *** $P < 0.001$

Table 17. ANOVA of fly blood-meal weights of teneral tsetses fed on ears previously exposed twice or 5 times a week

Source of variation	Sum of squares	DF	Mean square	F
Rabbits	277.6	1	277.6	5.21 *
Fly sex	2554.0	1	2554.0	47.92 ***
Interaction:				
Rabbit Fly sex	0.8	1	0.8	0.02
Residual	11777.4	221	53.3	
Total	14506.9	224	64.8	

Multiple classification analysis   Grand mean = 24.83 mg

Variable and category	Status	N	Deviation from mean (mg)
Rabbits:			
4 rabbits[a]	exposed 2 days/wk	174	0.48
1 rabbit - 3PD10	exposed 5 days/wk	51	- 1.63
Fly sex:			
Female		127	2.90
Male		98	- 3.76

Level of significance   * $P < 0.05$ *** $P < 0.001$

[a] Rabbits 5HB2, 5HB3, 5HB5, 5HB6

Table 18. Correlation between fly weight and blood-meal weight.

Right ear - naive Left ear - previously exposed

Rabbit	Fly sex	Rabbit ear	N	Pearson's Correlation coef[a]
5HB2	male	left	20	0.182
		right	22	0.529 **
	female	left	32	0.145
		right	34	0.344 *
5HB3	male	left	19	0.442 *
		right	18	0.489 *
	female	left	24	0.554 **
		right	21	0.080
5HB5	male	left	20	0.065
		right	19	0.139
	female	left	20	0.386
		right	20	0.092
5HB6	male	left	18	0.628 **
		right	18	0.342
	female	left	22	0.489 *
		right	21	0.809 **
3PD10	male	left	19	0.392
		right	18	0.550 *
	female	left	30	0.039
		right	26	0.077
			441	

---

[a] Correlation coefficients were calculated using fly weight vs blood-meal weight for each fly sex for each ear for each rabbit.

Total no. of cases = 20

Significant correlation coefficients:

Ear:		Fly sex:	
Left (previously exposed)	4	Male	5
Right (naive)	5	Female	4

Level of significance * $P < 0.05$ ** $P < 0.01$

meal (i.e. exposed or naive ear) (Table 18).

3.4.3.2 Probing frequencies

Probing frequencies of flies feeding on the right ear of naive rabbits were not significantly different from probing frequencies made on the right ear of previously-exposed rabbits (Table 19; Appendix C, Table C3). Male flies probed significantly more times than females (Table 19, 20; Appendix C, Table C3). Probing frequencies of teneral flies fed on the left ear and back of previously-exposed rabbits were not consistently different from probing patterns of flies on naive rabbits (triple interaction with ANOVA; Table 21).

Using mean fly-meal weights from fly-meal experiment 1 (conducted one month earlier) and mean probing frequencies made by flies on the right ear and back of 6 rabbits, a significant negative correlation was obtained between the number of probes and the weight of the blood meal (Table 22). There was no tendency for a significant correlation between fly meal weight and the number of probes to be related to fly sex or to a particular area of a rabbit (Table 22). Tsetses probed more frequently and obtained lower meal weights on the previously-exposed back than on a previously-exposed or naive, right ear. A ratio of the mean fly-meal weight over the mean number of probes was used as an estimate of how efficient flies are at obtaining a blood meal. Regardless of whether or not a rabbit had been previously-exposed, flies were significantly more efficient at obtaining a blood meal from the right ear than from the back (Fig. 50). There was no difference in the feeding efficiency between previously-exposed and naive rabbits. Female flies were significantly more efficient at obtaining a blood meal than male flies (Student's t, $P < 0.05$). Flies required significantly less effort to obtain a blood meal from the right ear than from the back (Student's t, $P < 0.05$; Fig. 50).

Table 19. ANOVA of the probing frequency of teneral tsetses placed on the right ear of previously-exposed and naive rabbits.

Source of variation	Sum of squares	DF	Mean square	F
Rabbits	0.13	1	0.13	0.59
Fly sex	1.21	1	1.21	5.29 *
Interaction:				
Rabbit fly sex	0.20	1	0.20	0.87
Residual	27.87	122	0.23	
Total	29.48	125	0.24	

Multiple classification analysis

Probes = $\sqrt{\text{Probes} + 0.5}$

Grand mean = 1.65 probes

Variable and category	Status	N	Deviation from mean (probes)
Rabbit group:			
3PD8 and 5FA4	previously exposed	47	- 0.05
4 rabbits[a]	naive	79	0.03
Fly sex:			
Female		66	- 0.09
Male		60	0.10

Level of significance * $P < 0.05$

[a] Rabbits 3PD10, 4AJ10, 5FB1, 5FB3

Table 20. Probing frequency of male and female teneral tsetses.

No. of probes	Frequency of flies								
	Male flies			Female flies			Total		
	N	Percent	Cum. %	N	Percent	Cum. %	N	Percent	Cum. %
0	6	3.0	3.0	10	4.5	4.5	16	3.8	3.8
1	95	48.2	51.2	125	55.8	60.3	220	52.3	56.1
2	23	11.7	62.9	36	16.1	76.4	59	14.0	70.1
3	14	7.1	70.0	15	6.7	83.1	29	6.9	77.0
4	11	5.6	75.6	8	3.6	87.6	19	4.5	81.5
5	6	3.0	78.6	5	2.2	88.9	11	2.6	84.1
6	7	3.6	82.2	4	1.8	90.7	11	2.6	86.7
7 - 10	19	9.6	91.8	13	5.8	96.5	32	7.5	94.2
11 - 14	8	4.0	95.8	5	2.2	98.7	13	3.0	97.2
15 - 18	3	1.5	97.3	3	1.3	100.0	6	1.4	98.6
19 - 24	5	2.5	99.8	0	0.0	100.0	5	1.1	99.7
Total = 421	197			224			421		

Table 21. ANOVA of the probing frequency of teneral tsetses placed on the left ear and shaved back of previously-exposed and naive rabbits.

Source of variation	Sum of squares	DF	Mean square	F
Rabbit	0.26	1	0.26	0.39
Area of rabbit	53.33	1	53.33	79.70 ***
Fly sex	5.13	1	5.13	7.6 **
Interaction:				
Rabbit Area of rabbit	0.29	1	0.29	0.44
Rabbit Fly sex	2.08	1	2.08	3.11
Area of rabbit Fly sex	0.45	1	0.45	0.67
3 way interaction:				
Rabbit, Area of rabbit, Fly sex	5.31	1	5.31	7.93 **
Residual	192.05	287	0.67	
Total	260.27	294	0.89	

Multiple classification analysis

Probes = $\sqrt{\text{Probes} + 0.5}$

Grand mean = 2.13 probes

Variable and category	Status	N	Deviation from mean
Rabbit group:			
3PD8, 5FA4, 3PD10	previously exposed	107	0.01
4AJ10, 5HB3, 5FB1	naive	188	- 0.01
Area of rabbit:			
Back		154	0.41
Left ear		141	- 0.45
Fly sex:			
Female		158	- 0.14
Male		137	0.16

Level of significance *** $P < 0.001$ ** $P < 0.01$

Table 22. Negative correlation between the number of times a fly probes and the meal size taken. Probes and meal weights were obtained with different flies in 2 different experiments.

Rabbit	Area of rabbit	Fly sex[a]	$\bar{x}$ no. of fly probes	$\bar{x}$ fly meal weight (mg)
5FB1	right ear	F	1.42 (12)	30.32 (11)
		M	1.00 (8)	22.74 (20)
	back	F	5.00 (14)	28.63 (19)
		M	8.50 (10)	20.78 (18)
5FA4	right ear	F	1.00 (8)	28.25 (11)
		M	1.58 (12)	22.78 (20)
	back	F	4.59 (17)	28.97 (17)
		M	6.80 (9)	22.82 (17)
4AJ10	right ear	F	1.33 (15)	28.64 (15)
		M	1.92 (12)	27.21 (15)
	back	F	6.33 (18)	18.71 (23)
		M	4.45 (20)	20.61 (16)
3PD8	right ear	F	1.06 (18)	28.74 (18)
		M	2.33 (9)	21.84 (12)
	back	F	2.12 (12)	28.74 (18)
		M	9.29 (14)	21.84 (15)
5FB3	right ear	F	1.40 (5)	25.09 (16)
		M	1.71 (7)	23.47 (13)
	back	F	3.25 (8)	24.30 (25)
		M	6.64 (11)	18.34 (12)
3PD10	right ear	F	2.63 (8)	27.50 (15)
		M	2.08 (12)	19.73 (13)
	back	F	3.40 (10)	23.40 (26)
		M	2.90 (10)	22.75 (14)

[a] Fly sex F = female M = male

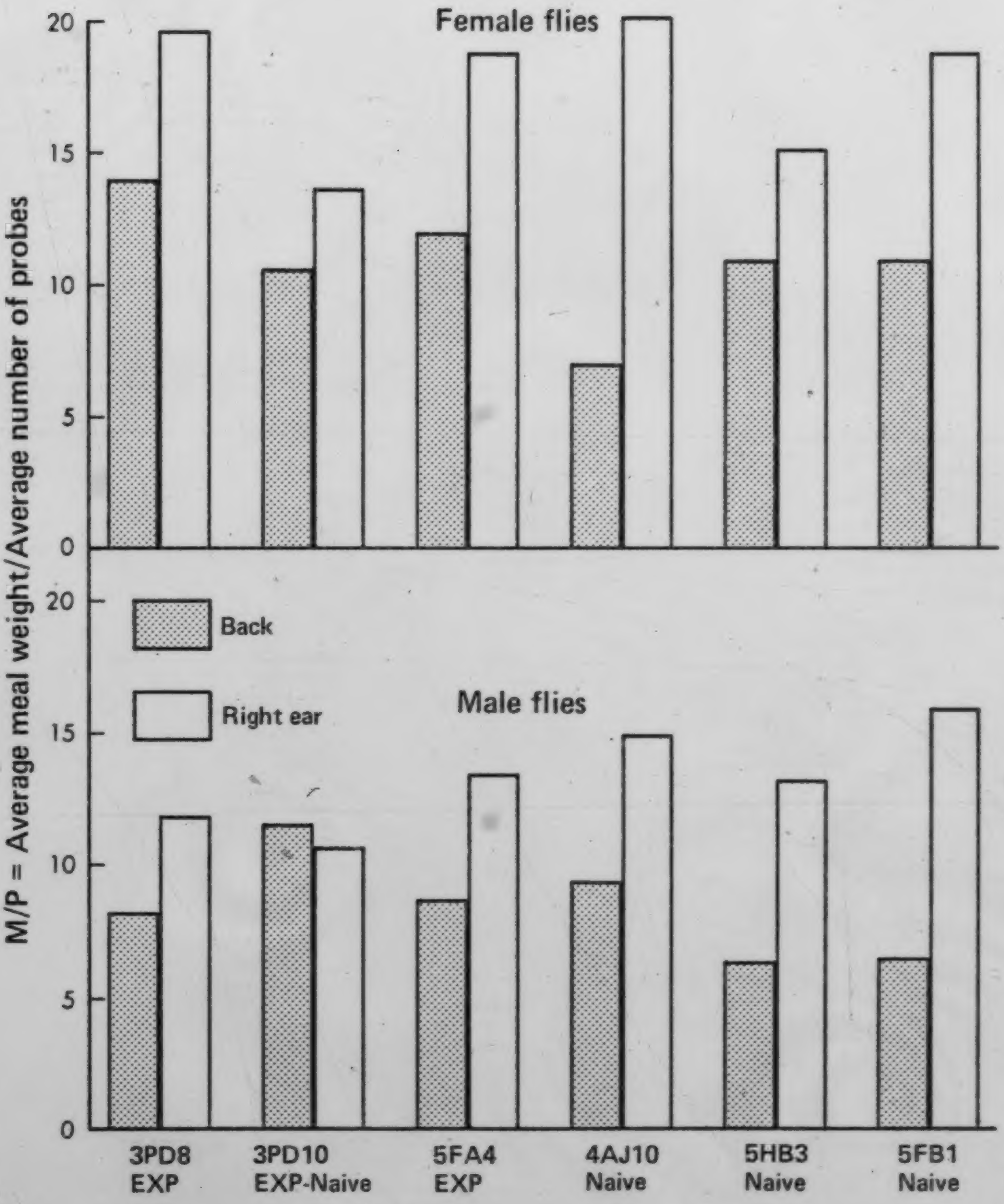
( ) = Number of flies used to calculate mean value

Spearman's correlation coefficients

Variable and category	Corr. coef.	No. cases
Right ear only	- 0.385	12
Back only	- 0.482	12
Females only	- 0.328	12
Males only	- 0.413	12
Females on back	- 0.371	6
Females on right ear	- 0.143	6
Males on back	- 0.371	6
Males on right ear	0.086	6
All 24 categories	- 0.448 *	24

* $P < 0.05$

Figure 50. Feeding efficiency of male and female teneral tsetses fed on the back and right ear of previously-exposed and naive rabbits. The higher the column, the more efficient a fly is at obtaining a blood meal, and the less effort required in doing so. Female flies were more efficient than males in obtaining a blood meal. There was no difference between the feeding efficiency of flies fed on naive rabbits. Flies were less efficient at obtaining a blood meal from the back than from the ear.



EXP = Previously Exposed

Higher values represent less effort or more efficiency.

Since flies are less efficient at obtaining a blood meal from the back than from the right ear, rabbits were examined individually for this trend. To examine the differential effort required to obtain a blood meal from the back versus the ear of the same rabbit, a ratio of the efficiency of obtaining a blood meal from the back over the efficiency to obtain a blood meal from the ear was used (Fig. 51). Only in rabbit 3PD10 did male flies have a ratio greater than 1, indicating that the effort required to obtain a blood meal from the ear was as difficult as from the back (Fig. 51). A ratio of 0.77, also the highest of the 6 rabbits examined, was also obtained for female flies fed on this rabbit.

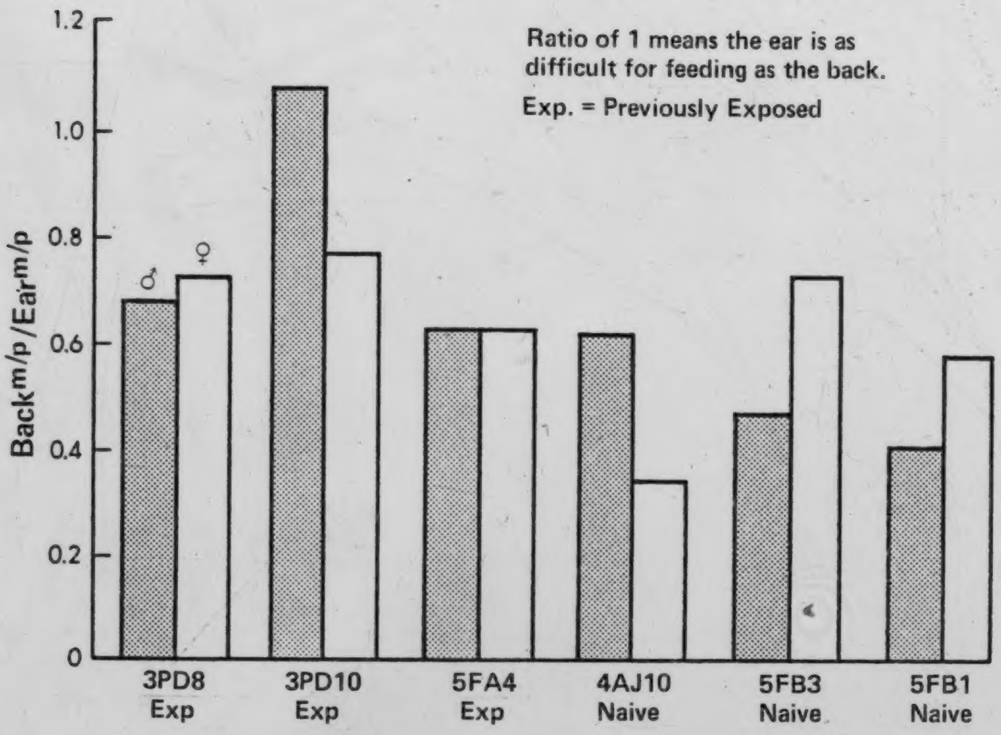


Figure 51. Feeding efficiency of teneral tsetses; comparing between the ear and back of the same rabbit. Ratios of how efficient flies obtain a blood meal were calculated ($\overline{x}$ meal wt/$\overline{x}$ probes) for the ear and the back (Fig. 50). This graph shows the difference in the feeding efficiency of flies between the ears and back of the same rabbit. Only in rabbit 3PD10 did male flies demonstrate a ratio greater than 1, indicating that the ear was as difficult a regime for feeding as the back. Female flies also fed on this rabbit demonstrated the highest back/ear ratio.

3.5 Discussion

Weights and haematological parameters were not significantly affected in rabbits exposed to 300 to 500 tsetses, 2 or 3 times a week. The same exposure, 6 days a week, resulted in decreases in rabbit haematocrits and to a less extent, changes in weights. The high level of exposure required to induce host response and the failure of host parameters to change dramatically, may be, in part, a result of the size of the rabbits used (4.7 - 6.2 Kg) and the high protein (18%) diet on which they were maintained. Through feeding a high protein diet, and eliminating stress to changing environmental conditions, these experimental rabbits would probably be able to withstand heavier biting-fly exposure than domestic or wild animals under natural conditions. In cases where host-diet has been deficient, reductions have been demonstrated in host growth, antibody production, haematopoiesis, and circulating globulins. Subsequently, these reductions in host response often result in a breakdown of resistance (see review by Nelson et al., 1977). Since antibody response in these rabbits developed quickly and responded strongly, and haematopoetic responses dropped to less than detrimental levels, host nutritional intake was probably more than sufficient to maintain metabolic processes, even during heavy tsetse exposure.

Host responses to ectoparasites include reductions in host weight and reductions in weight gains (review by Steelman, 1976; Campbell, 1976; review by Nelson et al., 1977). However, rabbits used for feeding 1000 to 1200 tsetses once a week at the Maison-Alfort Laboratory in France do not lose weight (Itard and Jordon, 1977). Similarly, in my experiments, rabbits exposed to tsetses 6 days a week did not show consistent weight losses, nor did rabbits exposed to 1200 to 1500 flies in a single, 4 hour period. Two of the three male rabbits exposed 6 days a week demonstrated almost no weight change, while the female rabbit gained weight. Of particular interest is the weight gain of 8.3% (760 g) in female rabbit 6AD8, while her male litter-mate, receiving the same exposure, lost as much as 11% (580 g) initial body weight. Furthermore, female rabbit 6DB4 had a sharp weight increase during the month following her first exposure to an estimated 1158 fly bites in 4 hours. This rabbit's female littermate, 6DB5, a rabbit receiving only control handling, increased its weight slightly. Growth curves for this cross of rabbit, and for most other closely related breeds, after 8 months of age, were not available (rabbit producers are interested mainly in the weight gain during the first 4 months of age). However, weights of the three control rabbits (Fig. 34) receiving only one week of exposure to 120 flies/day did not show any consistent increase or decrease in weight, reducing the possibility that the increase in weight was normal.

The tick, Boophilus microplus feeding on cattle maintained on high and low levels of nutrition, produced both an anorectic effect, caused by the feeding of the tick, and a specific effect, caused by the toxins of the tick saliva, on host metabolism (O'Kelly and Seifert, 1970; O'Kelly et al., 1971). Similarly, the increase in weights of these two female rabbits may be the result of tsetse salivary toxins affecting female, but not male, host metabolism; perhaps osmoregulation and water retention. Three other female rabbits used in the 4 hour heavy exposure experiment, one control and 2 exposed rabbits, demonstrated no such weight gains. The reasons for a weight gain in some female rabbits and not in others remains uncertain.

Three control rabbits, receiving only one week of tsetse exposure, lost 2 to 5 times the estimated weight loss attributed to the blood meals taken by the tsetses (estimated on the assumption that all flies fed and that rabbits did not compensate for this by increased food consumption) during the one week of exposure. This additional weight loss is probably the result of an anorectic response, although the effects of salivary toxins cannot be excluded.

The effects of ectoparasites on host-haematological and biochemical parameters have been an area of study largely neglected. Haematocrits of cattle progressively decrease under light, moderate, and heavy exposures to the louse, Haematopinus eurysternus (see Collins and Dewhirst, 1965). Similarly, rabbit red cell volume is negatively correlated with tick exposure (Jellison and Kohls, 1937). Apparently, no similar studies with biting-flies have been reported, although Riek (1954) has demonstrated a direct relationship between histamine levels in blood of horses and levels of Culicoides robertsi attack. In my experiments, haematocrit values of rabbits exposed to tsetses, 6 days a week over a 20 week period, correlated closely with estimated blood loss due to tsetses. A slight decrease in the weekly estimated blood loss due to tsetses resulted in an increase of 2 to 4 haematocrit percent in the rabbit.

Nelson and Haufe (unpubl., from review by Nelson et al., 1977) have apparently noted that anemia in cattle is not always correlated with louse numbers. Nelson et al. (1977) suggest that this result may indicate that a toxin is involved in the development and maintenance of louse-induced anemia in cattle. In the present study, rabbit haematocrit levels decreased with increased tsetse exposure. This is probably due to the blood taken by the tsetse, although a salivary toxin may also affect red cell production at a rate proportional to tsetse exposure.

Some correlation was observed between weekly weight-specific blood weight and weekly haematocrit, however this correlation was not consistently positive or negative. Although host anemia was not severe, haematocrits decreased from pre-exposure values of 40 to 46 percent, to as low as 29 to 31 haematocrit percent.

Van Handel (1962) suggested that perhaps the intermittent feeding of blood-sucking insects, and consequently the injection of their anti-coagulants into man, may contribute to a decrease in coronary thrombosis. In conjunction with his suggestion, and with the results of the tests on the tsetse anticoagulant made in the 2nd Chapter, clotting times of rabbits during heavy tsetse exposure were examined to determine if the tsetse anticoagulant grossly effects *in vivo* clotting times as it does *in vitro* clotting times. Whole blood in cattle made anemic by the louse, *Haematopinus eurysternus*, is reported to clot much more rapidly than whole blood of cattle which were louse resistant (low louse infestations) (Nelson et al., 1970). Similarly, whole blood clotting times of rabbits exposed to tsetses 2 to 3 times a week were less than control rabbits receiving no exposure, the results being statistically insignificant due to the small sample size. Although my results agree with the observations mady by Nelson et al. (1970), whole blood clotting times are not an accurate measurement of the status of the coagulation system. For this reason thrombin times were obtained for 4 rabbits receiving heavy exposure to tsetses 6 days a week for 20 weeks, and 3 control rabbits. No consistent or significant changes occurred in the thrombin times of the citrated plasma taken at weekly intervals from any of the 7 rabbits. The possibility that the tsetse anticoagulant affects the *in vivo* clotting times of rabbits seems unlikely for 3 reasons. First, the tsetse is a telmophage (Gordon and Crewe, 1948), feeding from a pool rather than directly from a blood vessel. This type of feeding would greatly reduce the probability that a significant amount of anticoagulant reaches the vascular system, although evidence of circulating antibodies in rabbit sera indicates that some salivary gland components do enter the bloodstream. Second, the tsetse sucks back much of the injected saliva with the blood meal (Lester and Lloyd, 1928), reducing the probability that a significant amount remains in the host. Third, foreign substances such as the tsetse anticoagulant which do reach the vascular system would probably be excreted by the kidneys, as hirudin is, within a short period of time following intravenous injection (Markwardt, 1958).

Although close examination of host cutaneous responses was not a part of this study, a few observations were made. With the exception of one rabbit (3PD9), bleeding of previously exposed rabbits from the marginal artery of the ear was no more difficult than bleeding naive rabbits. Following exposure, the ears of the rabbits exposed to tsetses did not appear to show signs of irritation or abnormality. No dry, scaly, or oedematous conditions, similar to those described in goats exposed to tsetses (Nash et al., 1965), were observed. Only in one rabbit, 3PD9, was bleeding difficult. This rabbit's ears became thick skinned and hard. Bleeding from the ear was difficult, even with the use of xylene, an irritant used to dilate ear veins. In all 6 rabbits exposed to 1200 to 1500 tsetses in a 4 hour period,bleeding immediately post-exposure was very difficult. Xylene failed to alleviate the problem. A definite pronounced arteriolar vasoconstriction was observed in both ears. Citrated whole blood which was collected, often clotted even after periods of prolonged mixing. Whether or not this clotting of blood was a result of unclean venipuncture or some type of coagulation defect remains uncertain, although the first suggestion seems more feasible.

Naturally produced circulating antibodies were identified by immunoelectrophoresis and passive haemagglutination in all of the previously-exposed rabbits that were examined. Precipitating antibodies have been demonstrated in rabbits and guinea pigs exposed to Aedes aegypti (Wilson and Clements, 1965) and in rabbits exposed to Rhodnius prolixus (Fox and Bayona, 1968). Therefore, this is the third time naturally produced precipitating antibodies have been observed in hosts exposed to insects. A maximum of 7 bands were observed using immunoelectrophoresis. Some of these bands may represent salivary secretion precursors obtained by homogenizing the whole salivary glands. An attempt to obtain salivary secretions from the crop of tsetses fed on saline-ATP solution, and thus avoid homogenizing the salivary glands, was only partially successful. The crop contents of 100 male tsetses following dialysis and concentration, provided a much weaker antigen than the emulsified glands. Only two poorly visible and indistinct bands, one on the cathode side and the other on the anode side of the antigen well, were observed. Although these bands appeared to contain more than one antigen-antibody complex, the experiment was not repeated because of the time and effort involved. No precipitating antibodies were produced when tsetse hindguts and midguts were substituted for SGS, indicating that the salivary gland antigens are either broken down by digestive enzymes or that the antigenic sites of the salivary components are in some way blocked. It is also possible that with guts from fed flies, the antigens had already reacted with antibody in the blood meal, and that no further reaction was possible. However, no precipitation occurred with guts from unfed flies either, reducing the latter possibility. Substituting Sephadex G-75 fractions of SGS as antigen, precipitating antibodies were observed in all fractions which eluted with a molecular weight of over 15,000. No precipitating antibodies were observed when substituting Sephadex fractions containing maximum anticoagulant activity for SGS. Although these rabbit antibodies have not been shown to have a protective function, the lack of antigenic material in fractions containing maximum anticoagulant activity may explain why anticoagulant activity was not affected following incubation with sera of rabbits containing antibodies to SGS (see Section 2.4.5). It is to the tsetses advantage not to have an anticoagulant with antigenic properties. As pointed out in a review by Nelson et al. (1977), the responsive host "exerts a great selective pressure on the parasite to modify their antigenic properties and reduce antigenic disparity between the host and parasite".

Passive haemagglutination tests quantitatively established changes in antibody titres of rabbits exposed to varying levels of tsetse exposure. The maximum titres obtained were higher than any previously published reports using either acarina or larval Hypoderma (see review by Nelson et al., 1977; Brossard, 1976; Boulard and Weintraub, 1973), although titres as high as 5,120,000 have been reported in rabbits injected with caddisfly extracts (see review by Shulman, 1967). Of major significance here is that antibodies are naturally produced by the host when exposed to tsetses. Use of artificial hosts has resulted in serological responses different from natural hosts. Hyperimmunization of rabbits with 1st instar Hypoderma larvae (Boulard and Weintraub, 1973) produced responses different from those reported in cattle infested with the same species (Robertson, 1964). The authors suggest that part of this difference may be reflected in the fact that 1st instar larvae grew normally in the rabbits but could not complete development to further instars. Although use of the tsetse as a biting-fly and use of laboratory rabbits as hosts may produce serological responses which differ from natural situations, these results should provide a guideline from which to work.

Passive haemagglutination titres were observed even when precipitating antibodies were not. These results are probably due to the relative sensitivity of the various techniques used (PHT can detect as little as 0.005 µg/AbN: Williams and Chase, 1968; Precipitin ring test as little as 1.0 µg/AbN: Campbell et al., 1970; Agar diffusion as little as 5 to 10 µg/AbN: Kabat and Mayer, 1961). Titres were first measureable after one week of tsetse exposure, a much shorter time (21 days) than required to detect titres in rabbits infested with 1st instar Hypoderma larvae (Boulard and Weintraub, 1973), but approximately the same period of time to produce titres in cattle exposed to Boophilus microplus (Brossard, 1976). Titres correlated with tsetse exposure to some extent, although rabbits were variable in their response. Brossard (1976) reported a positive causal relationship between antibody titre and resistance development. Unfortunately similar comparison with this research was not possible since rabbit titres often were too high to be recorded, and rabbits used in resistance experiments (Chapter 4) were interchanged. During a period of constant tsetse exposure, titres of most rabbits either remained stable or declined. In two of the rabbits exposed to tsetses, titres returned to zero after 16 to 20 weeks of no exposure. A pause in the exposure of tsetses to rabbits resulted in a drop in titres, although of no more than 4 doubling dilutions. Titre responses in most rabbits exposed to a constant level of tsetses were similar to responses described in rabbits infested with 1st instar Hypoderma larvae (Boulard and Weintraub, 1973).

Fly blood meal weights and probing frequencies were used in an attempt to measure localized skin resistance. In fly meal experiment 1 using meal weights from the right ear and back, no significant difference was observed between the meal weights obtained from previously-exposed and naive rabbits. Unfortunately, this result does not indicate whether or not fly meal weights reflect skin resistance prior to deciding that there is no difference in fly meal weights between previously-exposed and naive rabbits. There were no marked differences in the appearance of, or the effort required to bleed, ears of previously-exposed and naive rabbits. Female flies took significantly larger meals than male flies, agreeing with results obtained by previous authors (Glasgow, 1963; Nash, 1969). The back proved to be a more difficult area for feeding than either a previously-exposed or naive ear. Other authors (Evans, 1950; MacLeod, 1949) have observed very few keds (*Melophagus ovinus*) on the backs of sheep. Nelson (pers. commun) reports that keds find the back of grazing sheep unfavourable for feeding, however the backs of sheep in the winter or of sheep kept indoors are not so unfavourable. The backs of rabbits used in my experiments, although shaved, were still less favourable than ears. This may be the result of at least two factors. First, the ears of rabbits are a major source of heat loss (Hill and Veghte, 1976) and are consequently heavily vascularized, probably more so than the back. Under various temperature and stress conditions, the blood flow to the ears may be increased or decreased. The ears of rabbits during exposure (but not following exposure to 1200 to 1500 flies in 4 hours) were warm, indicating a substantial blood flow. Second, the vascular system along the back would be expected to be less extensive, since there are no major organs requiring heavy and large amounts of blood.

In the second experiment on fly meals, significantly larger meals were obtained from both the previously-exposed and naive ears of rabbits exposed to tsetses twice a week than from a rabbit exposed 5 days a week. Although with regard to the development of skin resistance, these results might be as expected, the age differential between the rabbits exposed twice a week and the rabbit exposed 5 days a week (9 months vs 29 months) would definitely affect results (Nelson and Qually, 1958). This may account for the significant difference between fly meal weights obtained from the naive ear of rabbits exposed twice a week and the naive ear of the rabbit exposed 5 days a week. The possibility that skin resistance contributes a significant difference in meal weights is unlikely, since there was no difference in the meal weights between the previously-exposed and naive ears of rabbits exposed twice a week, or the rabbit exposed 5 days a week. If the difference between the rabbits exposed twice a week and the rabbit exposed 5 days a week was not the result of an age differential, then resistance would have to be systemic, since the previously-exposed ear was no more difficult to obtain a blood meal from than the naive ear of the same age group of rabbits. Meal weights taken by flies correlated with fly weights in only 9 of 20 cases. Since there was no tendency for a significant correlation between fly weight and meal weight to be related to fly sex or to the source of the meal (exposed vs naive ear), the 11 cases which did not correlate are probably the result of large fluctuations in the meal size. Therefore, the variability of meal weights makes this an unsuitable parameter for measuring acquired skin resistance.

As might be expected, probings made by teneral tsetses correlated negatively with fly blood-meal weights. The results of probing counts agree with results obtained with fly meals. The number of probes fluctuates widely, although flies did probe more times on the back than on either a previously-exposed or naive ear. Male flies probed significantly more times than females. Using the mean blood-meal weight from one group of flies divided by the mean number of probes of a second group of flies as a measure of the efficiency of obtaining a blood meal, less effort was required to obtain a blood meal from the ear (either previously-exposed or naive), than from the back. Consistent with the results of fly meal weights, probing results demonstrated there was no significant difference in the efficiency of obtaining a blood meal between previously-exposed and naive rabbits. Female flies were more efficient at obtaining a blood meal than male flies. Using the efficiency ($\bar{x}$ meal wt/$\bar{x}$ no. probes) of obtaining a blood meal from the back over the efficiency of obtaining a blood meal from the ear, an estimate of the difference in effort required to obtain blood meals from the back and ear of the same rabbit was calculated. Only in one rabbit (3PD10), exposed 5 days a week to its left ear and back, was the ratio equal to 1. This ratio indicates that the essentially naive, right ear was as difficult an area for feeding as the previously-exposed back. These results indicate that the difficulty in obtaining a blood meal from this rabbit may be due to a systemic resistance or an age factor.

Rabbit 3PD8, another of the 6 rabbits used for these ratios, belonged to the same litter as rabbit 3PD10. Although rabbit 3PD8 had received the same exposure as rabbit 3PD10 for at least 2 months prior to the experiment (Table 9), it did not show back/ear patterns close to 1. Back/ear ratios for rabbit 3PD8 were higher than those of the other 4 rabbits indicating that the efficiency of flies may be related to age rather than to a systemic form of resistance.

CHAPTER 4

TSETSE RESPONSE TO HOST-RESISTANCE

4.1 Introduction

Establishing levels of acquired host-resistance requires close sequential examination of natural parasite populations (review by Nelson et al., 1977). Apart from environmental factors, a number of host parameters including age, sex, nutritional status, genetic history, grooming behavior and endocrine levels are known to influence the expression of resistance (see reviews by Nelson et al., 1975, 1977). Since acquired resistance in some host-ectoparasite systems is local (see review by Nelson et al., 1977), consideration must also be given to specific locations of previous host exposure. These factors make interpretation of fluctuations in ectoparasite populations, without adequate control measures, very difficult.

Observations, even on the basic stages of an ectoparasite's life cycle are very tedious, time-consuming, and often unrewarding tasks. For this reason, most authors have associated host-resistance with either a reduction in ectoparasite numbers, or a reduction in the number of ectoparasites reaching engorgement (Boese, 1974). Rarely have other ectoparasite parameters such as fecundity and egg production been examined (Riek, 1962; Hewetson, 1971, 1972; Sutherland and Ewen, 1974; Wagland, 1975). Most of the reports pertain to the responses of ticks being maintained on various breeds and crossbreeds of innately resistant cattle (Kemp et al., 1976; O'Kelly and Spiers, 1976; Gladney et al., 1973; review by Nelson et al., 1977). Aside from demonstrating that fewer sheep keds (*Melophagus ovinus*) survive or engorge on sheep which have acquired a natural resistance (Nelson and Bainborough, 1963) there appear to be no other reports of responses in flies maintained on naturally resistant hosts.

There have been 3 attempts to examine responses of flies maintained on hosts immunized with various fly tissues. Mortality of Anopheles stephensi was reported significantly higher in mosquitoes maintained on rabbits immunized with mosquito midgut antigen than in mosquitoes maintained on control rabbits or rabbits immunized with whole mosquito antigen (Alger and Cabrera, 1972). The increase in mortality was attributed to antibodies. Alger and Cabrera (1972) postulated that such antibody may interfere 1) by damaging cells in the mosquito digestive tract, 2) by inhibiting an enzyme, 3) through mechanical blockage of gut microvilli (decreasing the absorptive surface of the gut), or 4) by killing the bacterial flora of the gut. Although microcomplement fixation tests were positive, titres did not correlate with mortality rates of mosquitoes.

In correlation with the sites of interference suggested by the previous authors, serum inhibitors are known to inhibit digestive proteases from mosquitoes and sheep keds (Gooding, 1966, 1972b, 1974a; Huang, 1971a, 1971b). Although it should prove rewarding to determine if such inhibitors are responsible for fluctuations in ectoparasite populations, reports correlating serum inhibitor levels with ectoparasite response are lacking.

A significant decrease in fecundity, but no change in mortality, was observed in female Aedes aegypti maintained for 10 days on rabbits or guinea pigs immunized with whole mosquito antigen (Sutherland and Ewen, 1974). No decrease in fecundity was observed 19 days after the last injection, a result which Sutherland and Ewen (1974) attributed to less than effective levels of antibodies. Although these authors suggest that humoral antibodies may be interfering with the process of oogenesis or with mosquito ingestion and digestion of the blood meal, the presence of such antibodies was not demonstrated. The antibodies were suggested to be at least genus specific, since female Culex tarsalis were not affected when maintained on rabbits immunized with Aedes aegypti homogenate.

Mortality of Stomoxys calcitrans was significantly higher in flies maintained for 15 days on rabbits immunized with a mixture of adjuvant and any one of four fly tissues than in flies fed on a rabbit injected with adjuvant alone (Schlein and Lewis, 1976). Mortality was highest in those flies maintained on the rabbit immunized with fly thoracic muscles. When compared with flies maintained on the control rabbit, flies maintained on some of the immunized rabbits were also shown to have a higher incidence of leg paralysis, an unequal deposition of the endocuticle, and a reduction in post-emergence growth. Precipitating antibodies were non-specific, since mortality of Glossina morsitans was also significantly higher in flies maintained on a rabbit immunized with Stomoxys cuticle and adhering hypodermal cells or wing buds than in tsetses maintained on a control rabbit. Mortality of G. morsitans was not affected when flies were maintained on rabbits immunized with Stomoxys thoracic muscles or abdominal tissues. Unfortunately, Schlein and Lewis (1976) did not attempt to replicate their results as did Alger and Cabrera (1972) and Sutherland and Ewen (1974). Nevertheless, Schlein and Lewis (1976) attributed the increase in mortality of both fly species to antibodies interfering with the function and growth of fly tissues. Their suggestion that antibodies are involved is substantiated by the demonstration that antibodies (identified by immunofluorescence) in the sera of rabbits immunized with different tissues of Sarcophaga falculata, pass, immunologically unchanged, through the gut lining of this fly and attach to the respective fly tissue on which the rabbit was immunized (Schlein et al., 1976).

Valid comparison of the response of biting-flies maintained on immunized hosts with those maintained on naturally resistant hosts is difficult. Both the route of injection and the nature of the antigen are much different, and as may been seen in the following examples, the mere presence of antibodies does not predict the fate of the parasite. Mice immunized by repeated intraperitoneal injection of normal mosquito salivary glands or mosquito heads were protected from intraperitoneal sporozoite (Plasmodium berghei) challenge, but not from intravenous sporozoite challenge (Alger and Harant, 1976). Antibodies in the serum of a person solidly immune to typhus had no influence on any stage of Rickettsia prowazeki infection in Pediculus humanus, although the rickettsia excreted by such lice had detectable amounts of immunoglobulins on their surface (Boese et al., 1973).

4.2 Scope of this research

Few studies have gone beyond establishing the association of abnormal ectoparasite responses with the development of resistance. Consequently, little is known of how resistance manifests itself. At present, the research has been devoted to examining those ectoparasites which are closely associated with their hosts. Ironically, these are the same ectoparasites which are easiest to control chemically or biologically. Examining the responses of biting-flies which may be attributed to host-resistance introduces a much different situation. Biting-flies are usually in close association with their hosts for only short, intermittent periods. Most have the advantage of feeding quickly, are able to change hosts or positions on hosts rapidly, and usually do not require close association with the host for a prolonged part of their life cycle. Methods for controlling biting-fly infestations, similar to those used for other ectoparasites, are usually not feasible. Adulticiding is often impractical, expensive, and unsuccessful. Although larviciding has become more accepted as a means for control, it is expensive and has limitations.

The objective of this research was to examine the biological parameters (adult survivorship, pupal weights, female productivity, and emergence of both sexes from pupae) of the tsetse which are influenced through host-resistance in the hope of gaining a better understanding of the physiological systems affected. Although at the present time researchers are at the stage of examining the mechanisms of resistance for their biological significance, in the future, these mechanisms may be utilized as a means for control; if not directed against the ectoparasite itself, then perhaps directed to establishing higher resistance thresholds in the host. In either case, successful methods of control at the host level will probably be more feasible, and in the long run, less expensive.

4.3 Materials and methods

Maintenance of the colony was outlined in Section 1.5. The flies used in these experiments were maintained in the same way, except that feeding regimes were restricted. To ensure an equal age distribution between populations of flies, the same number of newly emerged flies were added to each population daily, for 3 to 7 days, until the full complement of flies was reached. Fifteen flies were kept in each cage, and the sexes kept separate except for one overnight mating period when females were at least 4 days old and males at least 10 days old. Tsetses were fed from the day of emergence, on selected rabbits or portions of rabbits. In each experiment, populations of tsetses were fed 6 days a week for 45 days (5, 9-day larviposition cycles), from the production of the first pupae by each population.

Control and experimental populations of flies were mated at the same time, usually between the 14th and 16th day post-emergence if both sexes of flies were used, and on the 4th day post-emergence (with at least 10 day old colony males) if only female flies were used. In those experiments using both sexes of flies (Exp. I, II, and III), control males were mated with control females and experimental males with experimental females. On the day of mating, male tsetses were placed with females in a 1:1 ratio for a period of 12 to 18 hours, the flies chilled on ice, the sexes separated, and the male flies returned to their respective cages.

In each experiment 4 parameters were recorded:

4.3.1 Adult survivorship: This is expressed as percent survival of the original number of flies. Mortality was recorded twice a week during the first 3 experiments and daily during Experiments IV and V. Since not all of the mortality curves were suitable for regression statistics, the following equation was used (suggested by R. Weingardt, Statistician, Dept. of Computing Sciences, University of Alberta):

$$\text{Ratio} = \frac{M_x - M_{x+1}}{N} = \frac{C1}{C2 \times N} \quad \text{where}$$

the ratio is a value representing the daily mortality rate between two successive mortality checks.

C = actual number of flies alive on a particular day mortality was taken. A vector of C values exists for each sex or each population of flies.

C1 = vector C with the elimination of the initial fly count.

C2 = vector C with the elimination of the last fly count.

N = number of days elapsed since mortality was last taken.

A vector of ratios was obtained for each population of flies. The vectors were statistically analyzed using Student's t tests or ANOVA.

4.3.2 Pupal weights: Plexiglass trays (Fig. 1) layered with sand were used to house cages containing either control or experimental flies. These trays were examined, and the sand sifted daily, for pupae. Pupae were then placed in labelled vials for 24 hours to stabilize pupal weights (during the first few hours following pupation, pupae lose weight through desiccation), and then individually weighed. Small pupae (less than 10 mg) and larvae which did not pupate, were not weighed, but marked as abortions and discarded. Average pupal weights of G. morsitans maintained on rabbits range from 23 to 30 mg (Mews et al., 1976; review by Laird, 1977). Although there is no set lower limit of pupal weights in which flies will not emerge, those pupae less than 20 mg are unlikely to. The number and percent of pupae in this category was recorded for each tsetse population, during each of the first 5 larviposition cycles. Occasionally, larvae would crawl half-way through the mesh of the cages and pupate. These pupae were usually heavier than average, however attempts to remove them often resulted in damaging the pupal case. Those pupae damaged were excluded from pupal weight calculations, but included in calculations for female productivity. For this reason, the number of pupae used in pupal weight and female productivity calculations are not always equal. Pupae were collected for the first 5, 9-day larviposition cycles (Appendix A, Fig. A1), starting from the day a pupae was first produced. Mean (± S.D. = standard deviation) pupal weights were plotted for each larviposition cycle. Student's t tests or ANOVA (SPSS) were used to compare weights produced by each population of flies, between and within larviposition cycles.

4.3.3 Female productivity: This is defined as the number of pupae (weighing over 20 mg) produced per female during each larviposition cycle. Values were obtained by dividing the number of pupae by the number of female flies surviving at the mid-point of each larviposition cycle. Female productivity was converted to $\sqrt{\text{Female productivity} + 0.5}$ and statistically analyzed using ANOVA (SPSS).

4.3.4 Emergence from pupae: This is expressed as a percent and defined as the total number of flies emerging from the number of pupae collected during each larviposition cycle. The sex ratio (usually 1:1) was also calculated and expressed as a percentage. Results were not statistically analyzed.

4.4 Experimental Design

4.4.1 Definitions:

NAIVE: Naive refers to a rabbit or portion of rabbit which has received no tsetse exposure prior to the start of an experiment. During an experiment naive rabbits, or portions of rabbits, received only tsetse exposure from a designated population of control flies.

PREVIOUSLY-EXPOSED: Previously-exposed refers to a rabbit or a portion of a rabbit which has received some tsetse exposure prior to the start of an experiment. During an experiment, previously-exposed rabbits or portions of rabbits receive exposure to a designated population of experimental flies as well as additional exposure to colony flies.

4.4.2 Experiment I (tsetse parameters affected by host-resistance)

Purpose: To establish which of the tsetse parameters outlined in Section 4.3 are affected by host-resistance.

Design: Three naive, control rabbits and one previously-exposed, experimental rabbit were used (Table 23). One population each of male and female tsetses (Table 23) were maintained on the three control rabbits, the other population of male and female flies were fed on the experimental rabbit. The three control rabbits were interchanged daily (each used twice a week) in an attempt to reduce the possibility of these rabbits developing resistance. Flies were fed on either ear or the shaved back of the rabbits without restrictions.

4.4.3 Experiment II (tsetse parameters affected by host-resistance)

Purpose: This experiment was designed to verify the results obtained in Experiment I, as well as to eliminate the possibility that age differential between control and experimental rabbits (present in Exp. I) may influence the results.

Design: Six naive, control rabbits and one previously-exposed, experimental rabbit were used (Table 24). The design of this experiment was similar to Experiment I, except that a third population of male and female flies were maintained on a naive rabbit from the same litter and therefore of the same

Table 23. Design of Experiment I (tsetse parameters affected by host-resistance).

Category and status	Rabbit I.D.	Rabbit[a] age (mo)	Rabbit sex	No. of flies male	No. of flies female
Control-naive rabbits	5EH3 5EH4 5EH5	6	male female female	127	120
Experimental - previously-exposed rabbit[b]	3PD9	22	male	123	120

[a] Age at the start of the experiment.

[b] Rabbit 3PD9 was exposed to colony tsetses 6 days a week for 16 weeks prior to this experiment. This rabbit also received exposure to colony tsetses throughout the experiment.

Table 24. Design of Experiment II (tsetse parameters affected by host-resistance).

Category and status	Rabbit I.D.	Rabbit[a] age (mo)	Rabbit sex	No. of flies	
				male	female
Control-naive rabbits	5HB2	5	male	120	120
	5HB3				
	5HB4				
	5HB5				
	5HB6				
Control-naive rabbit	3PD10	25	female	120	120
Experimental - previously-exposed rabbit[b]	3PD1	25	male	120	120

[a] Age at the start of the experiment.

[b] Rabbit 3PD1 was used to feed colony tsetses 6 days a week for 4 months during the 11th to 7th months prior to this experiment, and again for 3 weeks, from the 10th to the 7th weeks prior to this experiment. This rabbit received exposure to colony tsetses throughout the experiment.

age as the previously-exposed rabbit (Table 24). The experimental rabbit (3PD1) was exposed on both ears and the back to both colony and its population of experimental flies. The 6 naive rabbits had only their left ears and backs exposed to control populations of flies. The right ear of the naive rabbits received no exposure.

4.4.4 Experiment III (local or systemic resistance)

Purpose: To determine if acquired host-resistance is local or systemic.

Design: In Experiment II, the 6 originally naive rabbits had only their left ears and backs exposed to tsetses. At the completion of Experiment II, fly meal weight experiment 2 was performed (see Section 3.4.3.1). In fly meal experiment 2, meal weights from the previously-exposed, left ear were compared with those taken from the naive, right ear. From the results of the experiment, the 4 rabbits used in this experiment were chosen. The two rabbits with the greatest differences in meal weights between the previously-exposed and naive ears were selected as one pair. The two rabbits with the least differences in fly meal weights between the previously-exposed and naive ear were chosen as a second pair. Populations of male and female flies were maintained on the naive and previously-exposed ears of both pairs of rabbits (Table 25). If, at the end of Experiment III, there is a difference in the parameters of flies maintained between the previously-exposed and naive ears, it would be interpreted as a local rather than a systemic form of resistance.

4.4.5 Experiment IV (effect of naturally produced antibodies)

Purpose: To eliminate local resistance as a factor contributing to host-resistance, and to determine if circulating antibodies are detrimental to the tsetse.

Design: Seven naive rabbits and 4 previously-exposed rabbits were used

Table 25. Design of Experiment III (local or systemic resistance). Populations of male and female flies were maintained on the previously-exposed, left and the naive, right ears of each pair of rabbits.

Meal weight category (see Section 3.4.3.1)	Rabbit[a] I.D.	Rabbit[b] age (mo)	Rabbit sex	No. of flies fed on each ear male	No. of flies fed on each ear female
Least difference in fly meal weights between ears	5HB5	7	male	60	60
	5HB6	7	male		
Greatest difference in fly meal weights between ears	3PD10	27	female	90	90
	5HB3	7	male		

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[a] Each rabbit in a pair was used to feed the respective fly populations 3 consecutive days a week. Throughout the experiment, colony flies were fed on the left ear and back of all 4 rabbits in an attempt to maintain resistance in the left ear. Each of the four rabbits used had received exposure to their left ear and back for a period of 4 months prior to this experiment. The right ear had received no exposure (except for 20 - 40 flies used in fly meal weight determinations between the previously-exposed and naive ears). Although the rabbits were paired as greatest or least difference in fly meal weights between ears, this difference was never significant.

[b] Age at the start of the experiment.

(Table 26). The 4 previously-exposed rabbits had their left ears and backs (but never their right ears) exposed to colony tsetses, 6 days a week, for 4 months prior to this experiment. This experiment was designed to feed flies on the naive ears of naive and previously-exposed rabbits. Presumably, previously-exposed rabbits would have high antibody titres from tsetse exposure. Naive rabbits would have no antibodies. Groups of control and experimental rabbits were used so that rabbits could be interchanged at weekly intervals. For control rabbits, this would prevent the development of local resistance in the right ear as well as maintaining low antibody titres. For previously-exposed rabbits, interchanging would only reduce the development of local resistance in the right ear, since throughout the experiment, the experimental rabbits were exposed to colony tsetses on their left ears and backs to maintain high antibody titres. Previously-exposed rabbits were interchanged on a 4 week cycle (Table 26). Naive rabbits were interchanged on a 6 week cycle, with their second exposure being reduced to 3 days instead of 6 (Table 26). Attempts were made to match genetically previously-exposed and naive rabbits within the experiment by using litter-mates as the previously-exposed and control rabbits each week.

4.4.6 Experiment V (effect of naturally produced antibodies)

Purpose: In Experiment IV, the previously-exposed rabbits receiving exposure to both experimental and colony flies became anemic while control rabbits receiving only exposure to experimental flies did not. This experiment was designed to eliminate the possibility that anemia is a factor contributing to the results obtained in Experiment IV.

Design: Five naive, control rabbits and 4 previously-exposed, experimental rabbits (the same 4 used in Experiment IV) were used. This experiment

Table 26. Exposure schedule for Experiment IV (effect of naturally produced antibodies). A population of 120 female flies were maintained on each group of rabbits.

Week of experiment	Control (naive) rabbit used	Sex[a]	Experimental (previously-exposed) rabbit used[b]	Sex[a]
1	6AD9	F	6AD7	M
2	6AD10	F	6AD8	F
3	6AE2	M	6AE1	M
4	6CA3	M	6CA2	M
5	6AD11	F	6AD7	M
6	6AE3	F	6AE1	M
7	6AD9/6AD10[c]	F/F	6AD8	F
8	6CA3/6AE3	M/F	6CA2/6AE1	M/M
9	6AD11/6AD9	F/F	6AD7	M
10	6AD10/6AD5	F/F	6AD8	F

[a] Rabbit sex F = female M = male

[b] Experimental rabbits were exposed to the left ear and back by colony tsetses before and throughout the experiment.

[c] X/Y = where X and Y are rabbits exposed for 3 consecutive days during that week.

All of the rabbits used were between 6 and 8 months of age at the start of the experiment.

was designed in the same manner as Experiment IV, except that 1 week prior to the time a naive rabbit was scheduled to be exposed (Table 27), it was bled until haematocrit levels were similar to haematocrits of previously-exposed rabbits. All bleeding was done from the left ear. The experimental flies were maintained on the right ear. Tsetse populations were established similar to Experiment V (Table 27). Matching control and experimental rabbits genetically, by use of litter-mates, was not possible.

4.5 Results

4.5.1 Experiment I - Tsetse parameters affected by resistance

4.5.1.1 Adult survivorship

Female survivorship was significantly lower ($P < 0.05$) in flies maintained on the previously-exposed rabbit than in flies maintained on the 3 naive, control rabbits (Fig. 52). Male survivorship was not affected (Fig. 52). At the end of 57 days, survivorship was 27.8% lower in female flies and 18% lower in male flies maintained on the previously-exposed rabbit (Fig. 52).

4.5.1.2 Pupal weights

The pupae of flies fed on the previously-exposed rabbit were lighter 3 of 5, 9-day larviposition cycles than mean pupal weights obtained from flies maintained on the 3 naive rabbits (Fig. 53; Appendix D, Table D1). ANOVA, using the mean pupal weights of all 5 larviposition cycles, was not significant ($P > 0.05$), although pupae produced by flies maintained on the previously-exposed rabbit were 0.62 mg lighter than the overall average (Table 28). Flies fed on the naive and previously-exposed rabbits produced 2.9 and 5.0% pupae less than 20 mg respectively (Appendix D, Table D2).

Table 27. Exposure schedule for Experiment V (effect of naturally produced antibodies). Populations of female tsetses (n=120) were maintained on each group of rabbits. Rabbits were interchanged in an effort to prevent the development of skin resistance. Control rabbits were interchanged in order to reduce levels of antibody titres.

Week of experiment	Control (naive) rabbit used	Sex[a]	Experimental (previously-exposed) rabbit used[b]	Sex[a]
1[c]	6DB2	F	6AD7	M
2	6DB2	F	6AE1	M
3	6CD2	M	6AD8	F
4	6DB5/6DB3[d]	F/M	6CA2/6AE1[e]	M/M
5	6DB3	M	6AD7	M
6	6DB1	F	6AD8	F
7	6CB2	F	6AE1	M
8	6DB2	F	6CA2	M
9	6CD2	M	6AD7	M
10	6DB3	M	6AD8	F

[a] Rabbit sex F = female M = male

[b] Experimental rabbits were exposed to the left ear and back to colony tsetses in an attempt to maintain high antibody titres. Experimental flies were fed on the right (naive) ears of both control and experimental rabbits.

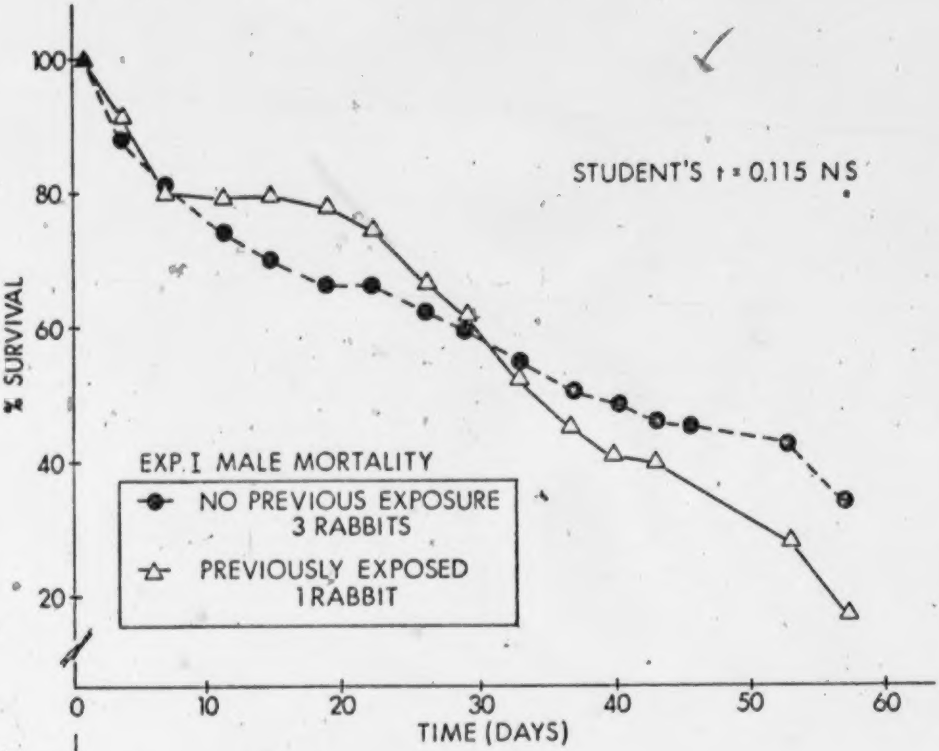
[c] During week 1, populations of tsetses were being established and exposure to rabbit 6DB2 was less than the full complement of 120 flies exposed to the same rabbit and subsequent rabbits after the first week.

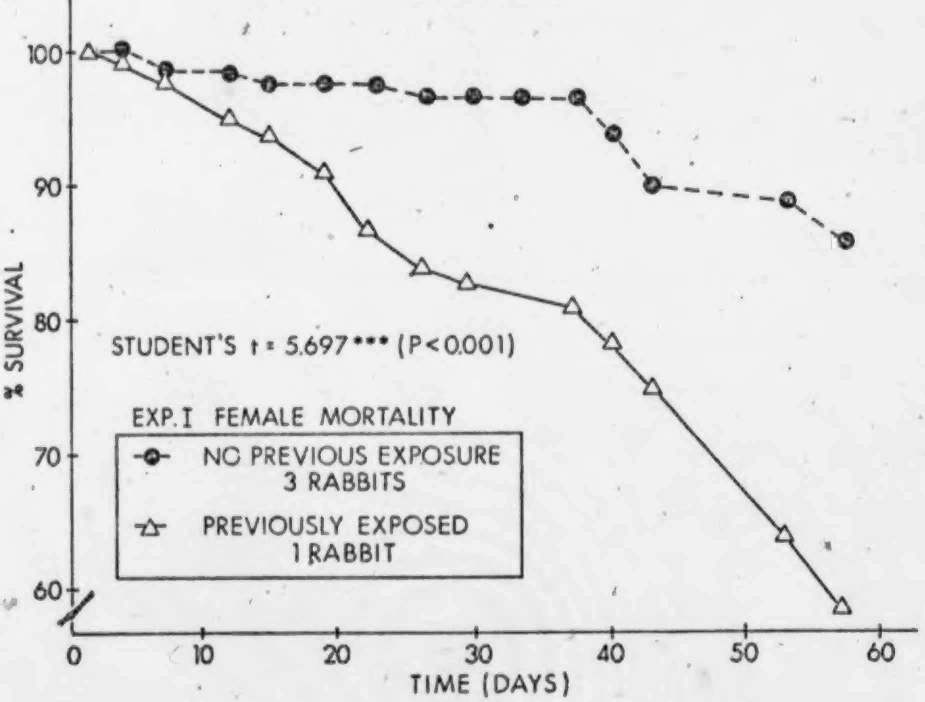
[d] Rabbit 6DB5 died of heart failure after 2 days exposure to tsetses. Death was presumably caused by excessive blood letting. Each control rabbit was bled so that haematocrit values in control and experimental rabbits would be similar.

[e] Experimental rabbits were interchanged to compensate for changes made with control rabbits.

Age in weeks of rabbits at the start of the experiment is as follows:
6AE, 6AD: 49; 6CA: 41; 6DB: 30; 6CD, 6CB: 36

Figure 52. Survival curves of flies used in Experiment I (tsetse parameters affected by host-resistance). Student's t tests were performed using vectors of a mortality ratio (see Section 4.3.1) representing the daily mortality rate between two successive mortality checks. Female survivorship was significantly lower in those flies maintained on the previously-exposed rabbit. Male survivorship was not affected.


STUDENT'S t = 0.115 NS
% SURVIVAL
100
80
60
40
20
EXP. I MALE MORTALITY
NO PREVIOUS EXPOSURE 3 RABBITS
PREVIOUSLY EXPOSED 1 RABBIT
0
10
20
30
40
50
60
TIME (DAYS)


% SURVIVAL
100
90
80
70
60
STUDENT'S t = 5.697*** (P<0.001)
EXP. I FEMALE MORTALITY
NO PREVIOUS EXPOSURE 3 RABBITS
PREVIOUSLY EXPOSED 1 RABBIT
0
10
20
30
40
50
60
TIME (DAYS)

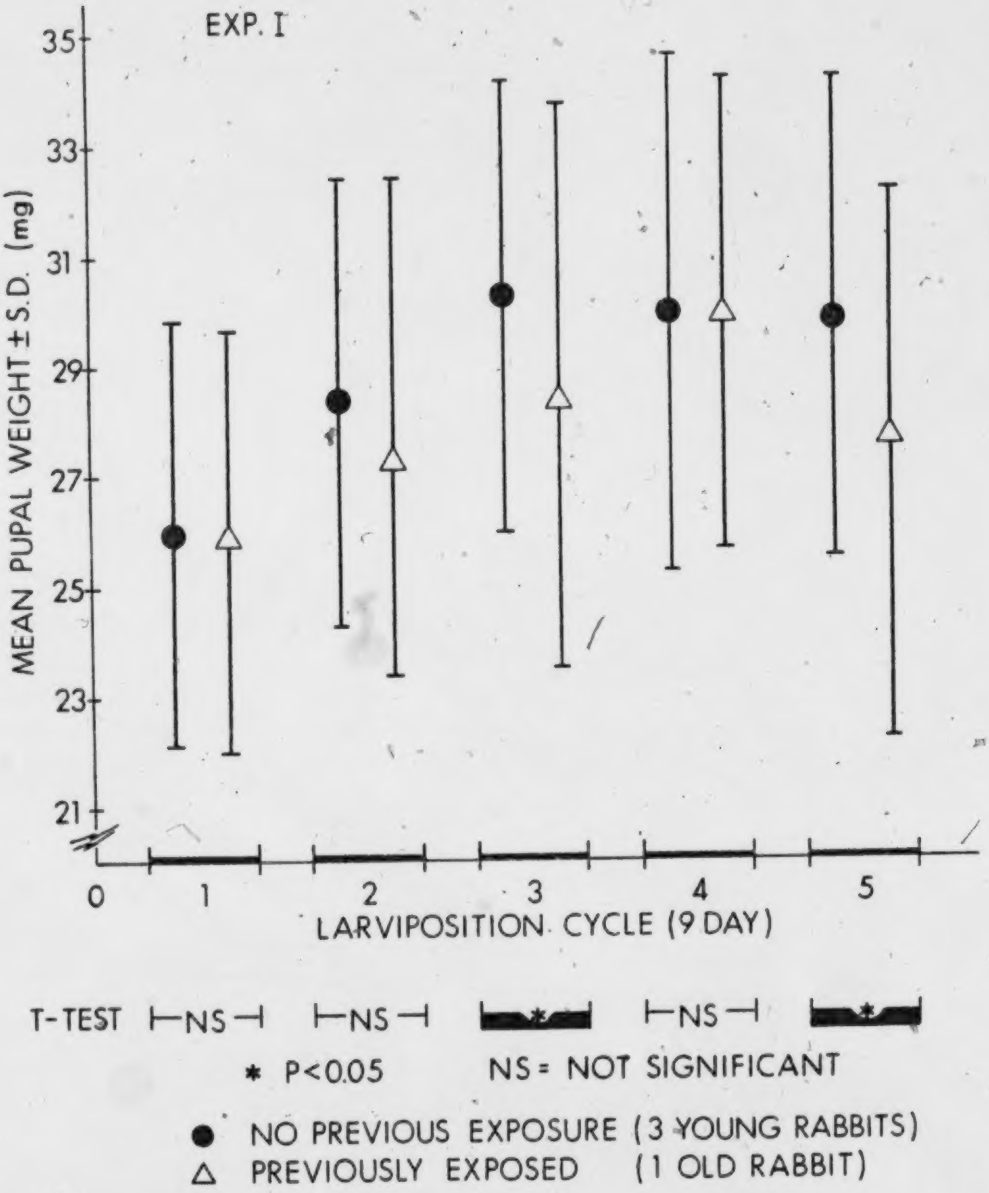


Figure 53. Pupal weights obtained from flies used in Experiment I (tsetse parameters affected by host-resistance). Pupal weight means in 3 of the first 5 larviposition cycles were lower in flies maintained on the previously-exposed rabbit.

Table 28. ANOVA of pupal weights obtained from flies used in Experiment I (tsetse parameters affected by host-resistance).

Source of variation	Sum of squares	DF[a]	Mean square	F
Between rabbits[b]	3.807	1	3.807	1.273
Residual	23.928	8	2.991	
Total	27.734	9	3.082	

[a] ANOVA was calculated using the mean pupal weight of each population of flies over the first 5 larviposition cycles.

[b] Rabbit groups = 3 naive, control rabbits; 1 previously-exposed rabbit.

Multiple classification analysis:

Grand mean = 28.34 mg Analysis includes pupae less than 20 mg weight

Variable	Status	No. of cycles	Deviation from mean (mg)
Rabbit group:			
3 naive young rabbits	naive	5	0.62
1 old rabbit	previously-exposed	5	- 0.62

4.5.1.3 Female productivity

Total female productivity was significantly lower ($P < 0.05$) in flies maintained on the previously-exposed rabbit (Fig. 54, Table 29; Appendix D, Table D2).

4.5.1.4 Emergence from pupae

There was no difference in the percent of flies which emerged from pupae produced by either group of flies (Table 30).

4.5.2 Experiment II - Tsetse parameters affected by resistance

4.5.2.1 Adult survivorship

Similar to the results obtained in Experiment I, female flies maintained on the previously-exposed rabbit had a lower survivorship than flies maintained on either control group (Fig. 55). Male and female survivorship were not significantly ($P > 0.05$) affected, although the F ratio for the female flies was 2.53 (significant at $P < 0.10$). Female survivorship of flies maintained on the naive rabbits after 65 days was 22 and 30% less than survivorship of flies maintained on the previously-exposed rabbit (Fig. 55).

4.5.2.2 Pupal weights

In all 5 larviposition cycles, mean pupal weights were lower in flies maintained on the previously-exposed rabbit than in flies maintained on either control group (Fig. 56, Appendix D, Table D3). The difference was statistically significant ($P < 0.05$; Table 31). Pupae less than 20 mg represented 4.1 and 4.8% of the pupae collected from flies fed on the naive rabbits and 7.6% of the pupae collected from flies fed on the previously-exposed rabbit (Appendix D, Table D4).

4.5.2.3 Female productivity

Female productivity among flies maintained on previously-

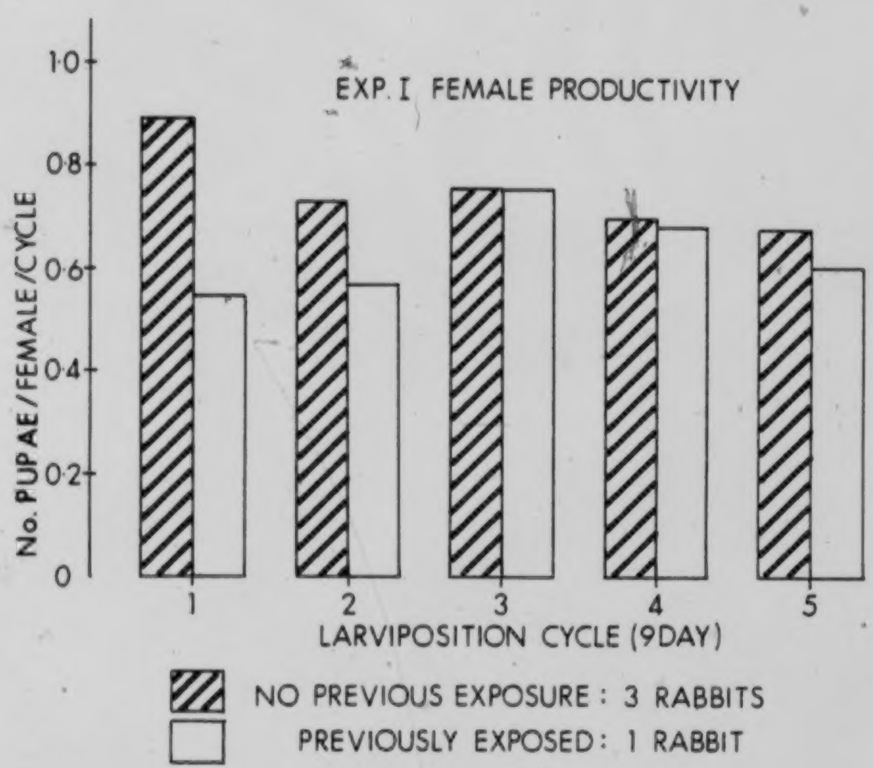


Figure 54. Female productivity of the flies used in Experiment I (tsetse parameters affected by host-resistance). Female productivity was significantly lower ($P < 0.05$) in those flies maintained on the previously-exposed rabbit. Analysis includes only those pupae over 20 mg weight.

Table 29. ANOVA of female productivity of flies used in Experiment I.

Source of variation	Sum of squares	DF	Mean square	F
Between rabbits	0.019	1	0.019	6.620 *
Residual	0.023	8	0.003	
Total	0.042	9	0.005	

Multiple classification analysis

Grand mean = 1.31 Female productivity = $\sqrt{\text{Prod.} + 0.5}$

Variable	Status	No. of cycles	Deviation from mean
Rabbit			
3 young rabbits	naive	5	0.04
1 old rabbit	previously and presently exposed	5	- 0.04

Level of significance * $P < 0.05$

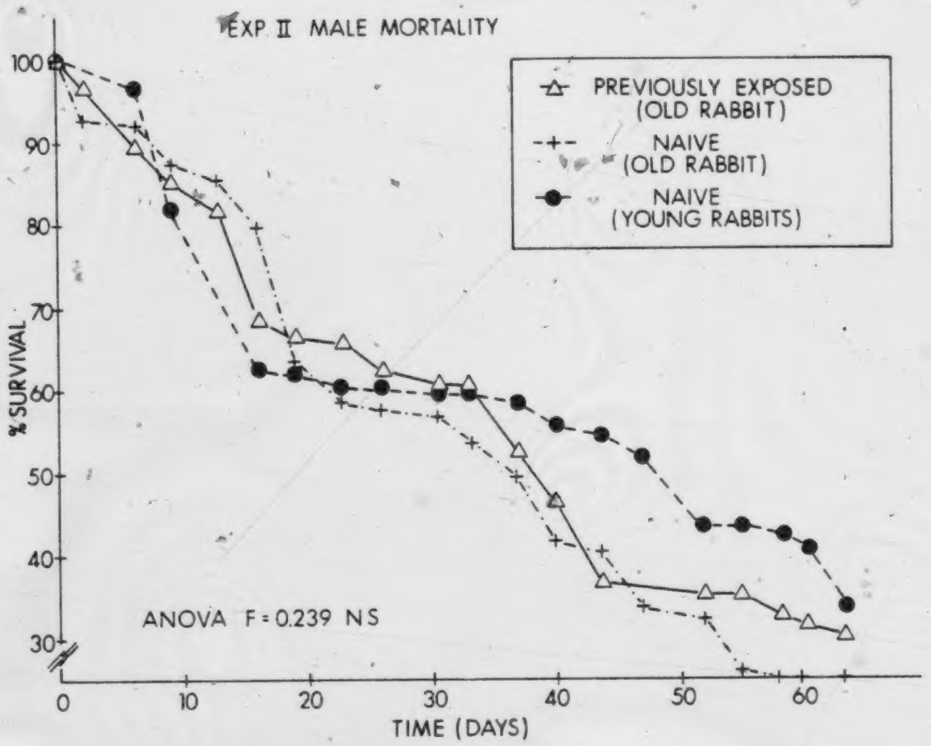
Analysis includes only those pupae over 20 mg weight.

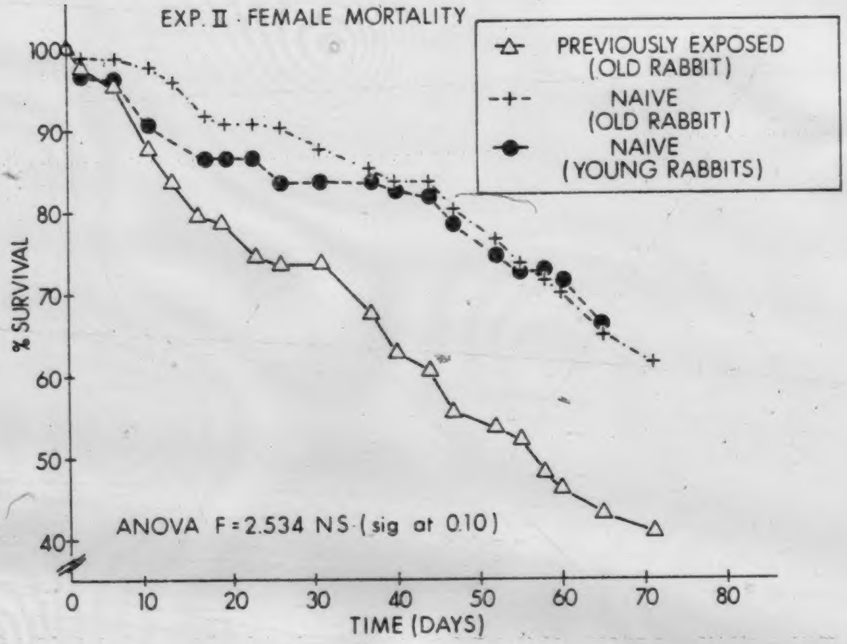
Table 30. Emergence from pupae collected during the first 5 larviposition cycles in Experiment I (tsetse parameters affected by host-resistance), YR = pupae from flies fed on the 3 naive control rabbits. PD9 = pupae from flies maintained on previously-exposed rabbit 3PD9.

Time [a] (weeks)	No. of pupae		No. of flies to emerge		No. of female flies to emerge		Percent emergence	
	YR	PD9	YR	PD9	YR	PD9	YR	PD9
1	45	26	34	22	17	11	75.5	84.6
2	75	57	62	33	31	14	82.7	57.9
3	79	39	57	37	27	28	72.1	94.9
4	45	58	24	42	13	17	53.3	72.4
5	53	46	39	40	20	21	73.6	86.9
6	61	39	49	24	28	11	80.3	61.5
7	65	34	57	30	34	22	87.6	88.2
Total	423	299	322	229	170	124	76.1	76.5

[a] Pupae were grouped by week rather than by larviposition cycles in this experiment only.

Figure 55. Survival curves of flies used in Experiment II (tsetse parameters affected by host-resistance). Male survivorship was not significantly affected when flies fed on a previously-exposed rabbit. Female survivorship was lower, although not significantly ($P > 0.05$; ANOVA calculated using vectors of ratios - see Section 3.4.1), in flies maintained on the previously-exposed rabbit. These rabbits are similar to those obtained in Experiment I (Fig. 52).


EXP. II MALE MORTALITY
PREVIOUSLY EXPOSED (OLD RABBIT)
NAIVE (OLD RABBIT)
NAIVE (YOUNG RABBITS)
% SURVIVAL
100
90
80
70
60
50
40
30
ANOVA F = 0.239 NS
0
10
20
30
40
50
60
TIME (DAYS)


EXP. II FEMALE MORTALITY
PREVIOUSLY EXPOSED (OLD RABBIT)
NAIVE (OLD RABBIT)
NAIVE (YOUNG RABBITS)
% SURVIVAL
100
90
80
70
60
50
40
ANOVA F = 2.534 NS (sig at 0.10)
0
10
20
30
40
50
60
70
80
TIME (DAYS)

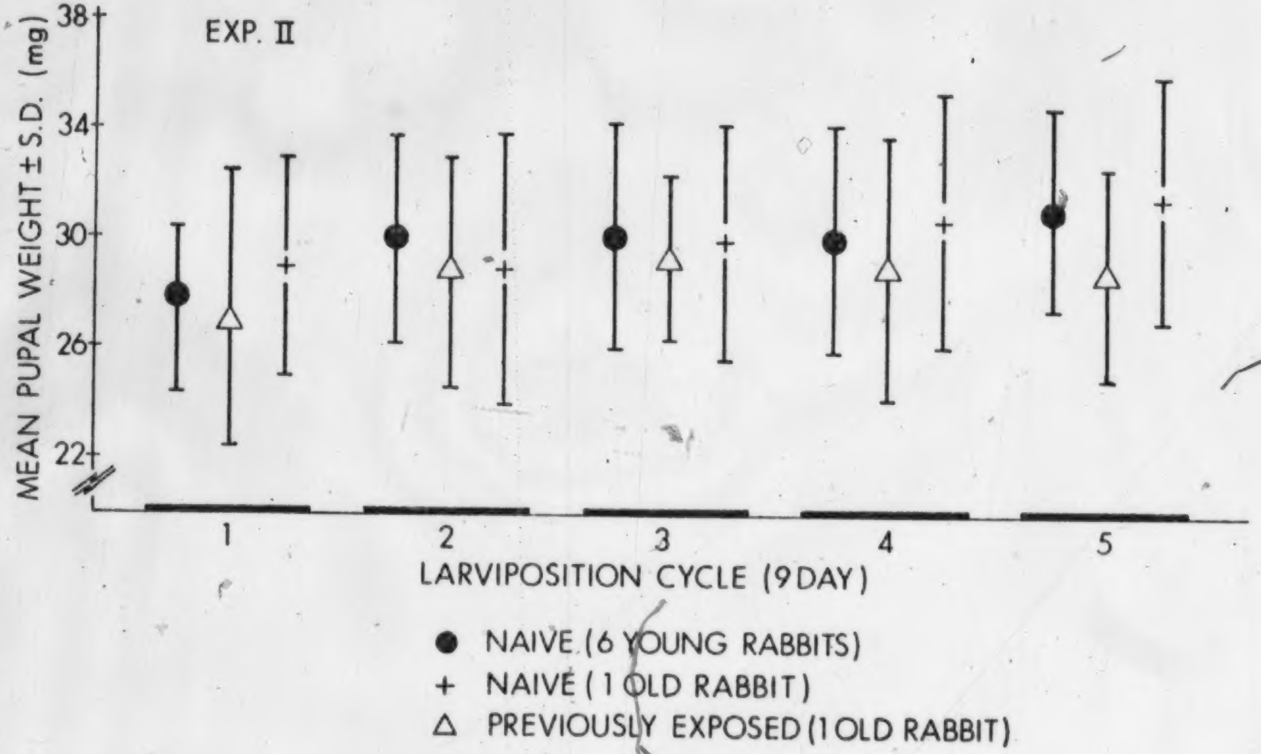


Figure 56. Pupal weights obtained from flies used in Experiment II (tsetse parameters affected by host-resistance). Pupal weights were significantly ($P < 0.05$) lower from the flies maintained on the previously-exposed rabbit.

Table 31. ANOVA of pupal weights from flies used in Experiment II.

Source of variation	Sum of square	DF	Mean square	F
Between rabbits	13.138	2	6.569	5.630 *
Residual	14.001	12	1.167	
Total	27.138	14	1.938	

Multiple classification analysis

Grand mean = 28.76 mg Analysis includes those pupae less than 20 mg.

Variable	Status	No. of cycles	Deviation from mean (mg)
rabbit used to feed flies:			
6 young rabbits	naive	5	0.56
1 old rabbit	naive	5	0.76
1 old rabbit	previously and presently exposed	5	- 1.32

Level of significance * $P < 0.05$

exposed and naive rabbits was not significantly different ($P > 0.05$; Fig. 57, Table 32), however flies maintained on the group of naive rabbits had a significantly higher productivity than flies fed on one naive or previously-exposed rabbit (Fig. 57, Table 32).

4.5.2.4 Emergence from pupae

Emergence of flies from pupae collected during the first 5 larviposition cycles was the same regardless of whether the flies fed on previously-exposed or naive rabbits (Table 33). Female flies accounted for approximately half of the flies emerged (Table 33).

4.5.3 Experiment III - Local or systemic resistance

4.5.3.1 Adult survivorship

Rabbits 5HB5 and 5HB6: The rabbits from which flies took the least difference in meal weights between the previously-exposed and naive ears.

Statistically ($P < 0.05$), male flies had significantly lower survivorship when fed on the previously-exposed ear, and females, a significantly lower survivorship when fed on the naive ear (Fig. 58). However, survival curves for both male and female flies (Fig. 58) differ very little between the naive and the previously-exposed ear, except for the two week period prior to mating (mortality of male flies normally increases for a short period after mating). During this time, the population of males feeding on the previously-exposed ear decreased sharply while those feeding on the naive ear did not. Using statistical analysis for this type of curve is difficult, since long periods of time with no change in mortality magnify shorter periods of time with change. At the end of 63 days, survivorship for both sexes of flies fed on either ear were similar (Fig. 58).

Rabbits 3PD10 and 5HB3: The rabbits from which flies took the greatest difference (although not statistically significant) in meal weights between

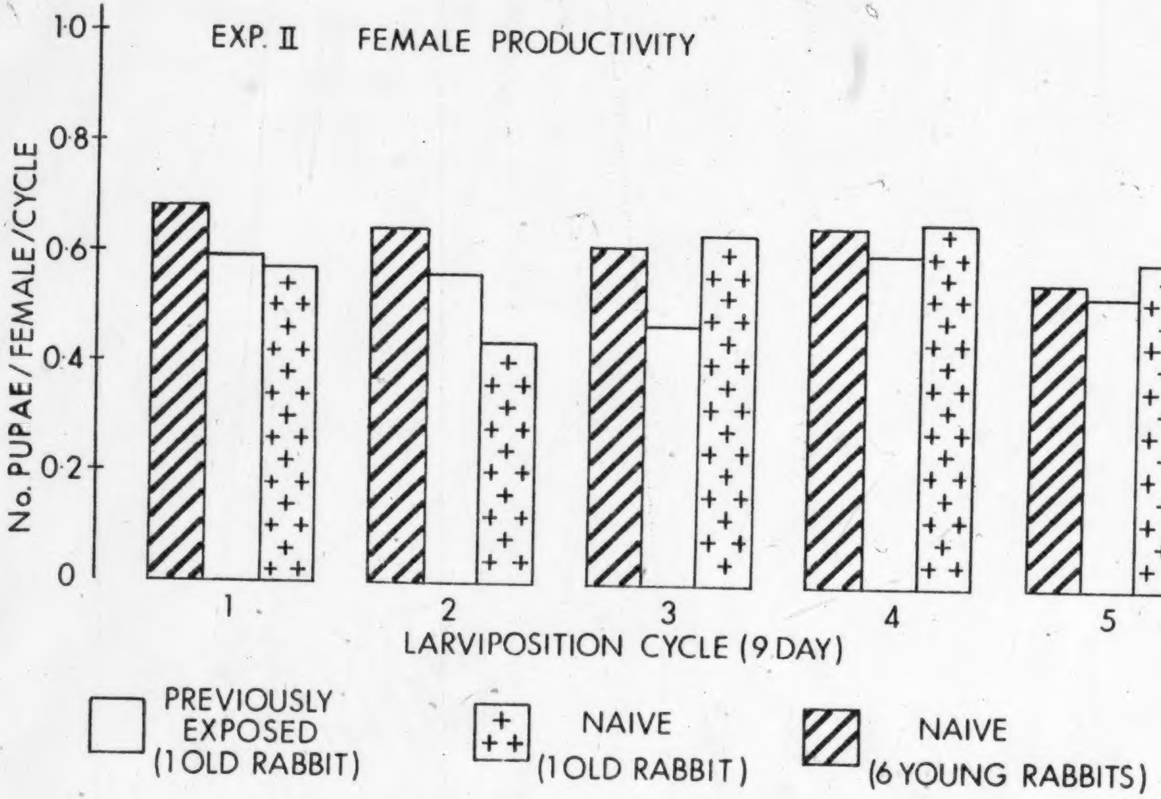


Figure 57. Female productivity of the flies used in Experiment II (tsetse parameters affected by host-resistance). Female productivity was not reduced when flies were maintained on a previously-exposed rabbit. Analysis includes only those pupae over 20 mg weight.

Table 32. ANOVA of female productivity of flies used in Experiment II (tsetse parameters affected by host-resistance).

Source of variation	Sum of squares	DF	Mean square	F
Between rabbits	0.006	2	0.003	2.911
Residual	0.012	12	0.001	
Total	0.017	14	0.001	

Multiple classification analysis

Grand mean = 1.27 Female productivity = $\sqrt{\text{Prod.} + 0.5}$

Variable	Status	No. of cycles	Deviation from mean
Rabbit used for feeding:			
6 young rabbits	naive	5	0.03
1 old rabbit	naive	5	- 0.01
1 old rabbit	previously and presently exposed	5	- 0.01

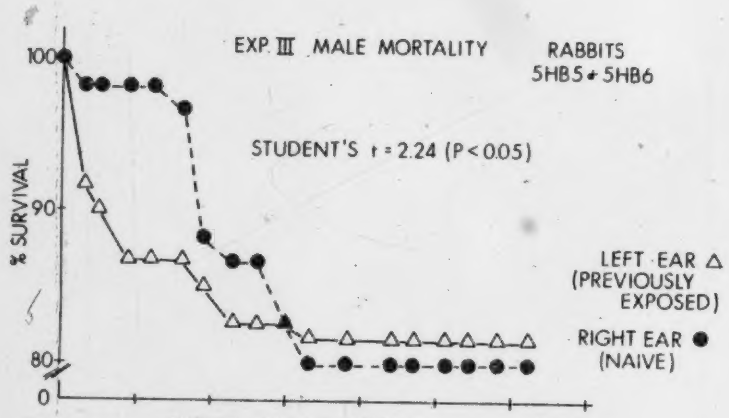
Analysis includes only those pupae over 20 mg weight.

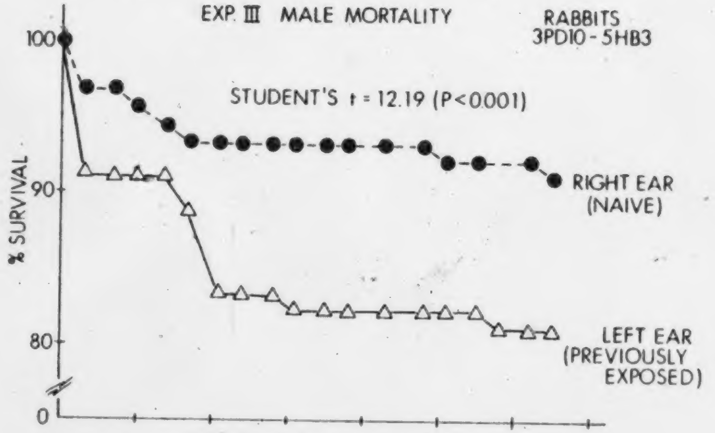
Table 33. Emergence from pupae collected during Experiment II (tsetse parameters affected by host-resistance). YR = flies fed on 6 young, naive rabbits. 3PD10 = flies fed on naive rabbit 3PD10. 3PD1 = flies maintained on previously-exposed rabbit 3PD1.

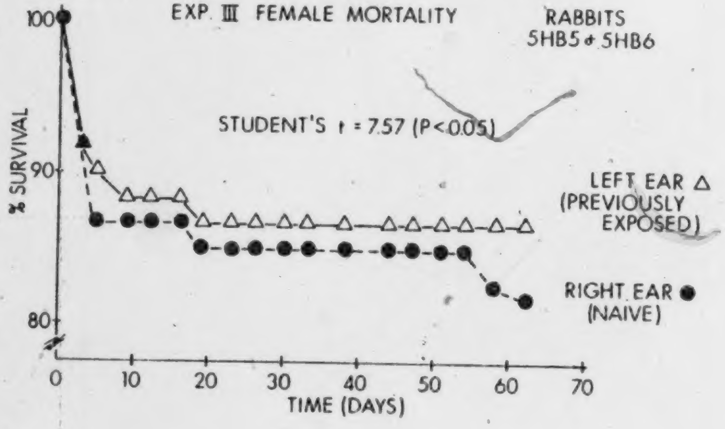
Larviposition cycle	Fly group	No. pupae over 20 mg	No. flies emerged Total	No. flies emerged Female	Percent emergence
1	YR	68	60	29	88
	3PD10	54	42	22	78
	3PD1	57	48	21	84
2	YR	64	57	28	89
	3PD10	60	49	31	82
	3PD1	45	40	17	89
3	YR	62	46	25	74
	3PD10	61	45	18	74
	3PD1	36	32	15	89
4	YR	60	51	24	85
	3PD10	53	44	22	83
	3PD1	39	32	18	82
5	YR	49	39	19	80
	3PD10	46	42	10	91
	3PD1	31	25	10	81
All 5 cycles	YR	303	248	125	82
	3PD10	277	223	113	81
	3PD1	208	177	81	85

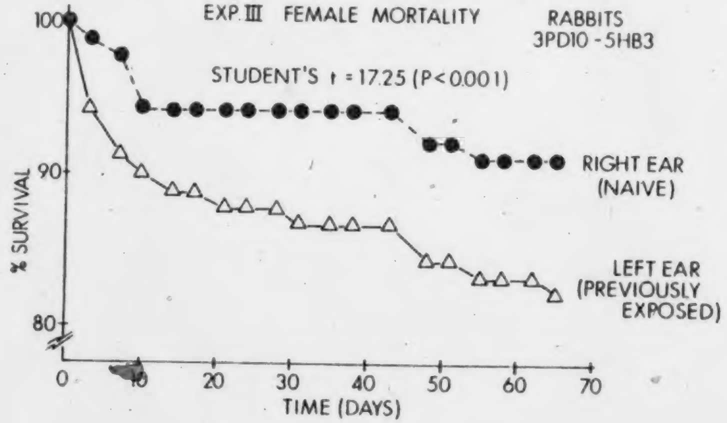
Figure 58. Survival curves of the flies used in Experiment III (local or systemic resistance). In the pair of rabbits with the least difference in fly blood-meal weights between ears (5HB5 - 5HB6), most of the mortality occurred in the two week period prior to mating. At the end of 65 days, survivorship in both males and females was essentially the same.

In the pair of rabbits with the greatest difference in fly blood-meal weights between ears (3PD10 - 5HB3), both male and female survivorship were 10% lower after 65 days, in the flies fed on the previously-exposed ear.


EXP. III MALE MORTALITY
RABBITS 5HB5 + 5HB6
STUDENT'S t = 2.24 (P < 0.05)
% SURVIVAL
100
90
80
0
LEFT EAR △ (PREVIOUSLY EXPOSED)
RIGHT EAR ● (NAIVE)


EXP. III MALE MORTALITY
RABBITS 3PD10 - 5HB3
STUDENT'S t = 12.19 (P < 0.001)
% SURVIVAL
100
90
80
0
RIGHT EAR (NAIVE)
LEFT EAR (PREVIOUSLY EXPOSED)


EXP. III FEMALE MORTALITY
RABBITS 5HB5 + 5HB6
STUDENT'S t = 7.57 (P < 0.05)
% SURVIVAL
100
90
80
LEFT EAR △ (PREVIOUSLY EXPOSED)
RIGHT EAR ● (NAIVE)
0
10
20
30
40
50
60
70
TIME (DAYS)


EXP. III FEMALE MORTALITY
RABBITS 3PD10 - 5HB3
STUDENT'S t = 17.25 (P < 0.001)
% SURVIVAL
100
90
80
RIGHT EAR (NAIVE)
LEFT EAR (PREVIOUSLY EXPOSED)
0
10
20
30
40
50
60
70
TIME (DAYS)

the previously-exposed and naive ears.

After 60 days, survivorship was 10% lower in both male and female flies maintained on the previously-exposed ears. Using statistical analysis (Student's t with vectors of ratio; see Section 4.3.1), this difference was significant ($P < 0.05$; Fig. 58).

4.5.3.2 Pupal weights

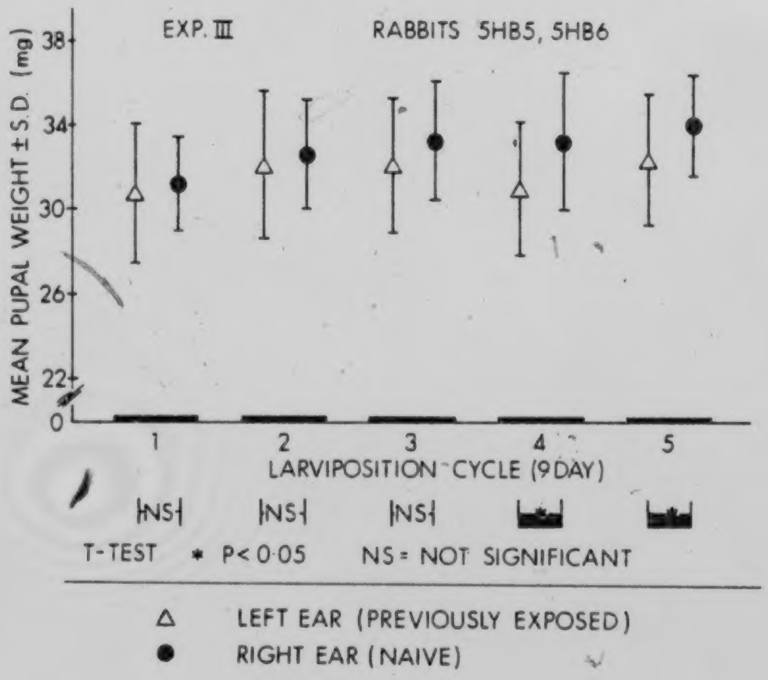
The pupae of flies fed on the naive ears of rabbits 5HB5 and 5HB6 were heavier in all 5 larviposition cycles than the pupae obtained from flies maintained on the previously-exposed ears (Fig. 59). In the last two larviposition cycles, the difference in pupal weights was significant ($P < 0.05$) between ears (Appendix D, Table D5). ANOVA, using all 5 cycles, was slightly short of significance ($P > 0.05$, Table 34). No pupae less than 20 mg weight were produced by either fly population fed on rabbits 5HB5 and 5HB6 (Appendix D, Table D6).

Weights of pupae produced by flies maintained on the naive ears of rabbits 3PD10 and 5HB3 were also heavier in all 5 larviposition cycles than pupal weights obtained from flies maintained on the previously-exposed ears (Fig. 59). Weights of pupae produced by flies maintained on the naive and exposed ears were significantly different ($P < 0.05$) in all but the second cycle (Appendix D, Table D5). ANOVA, using all 5 cycles, was also significant ($P < 0.05$; Table 35). Of the pupae collected from the flies maintained on rabbits 3PD10 and 5HB3, 1.5% from flies fed on the naive ears, and 5.6% from flies maintained on the previously-exposed ears, were less than 20 mg weight (Appendix D, Table D6).

4.5.3.3 Female productivity

In both pair of rabbits, female productivity was not affected when flies were maintained on the previously-exposed ears (Fig. 60, Table 36, 37; Appendix D. Table D6).

Figure 59. Pupal weights of flies used in Experiment III (local or systemic resistance). Regardless of whether flies fed on the previously-exposed ear of the pair of rabbits with the greatest (3PD10 - 5HB3) or least (5HB5 - 5HB6) differences in meal weights between ears, pupal weights were heavier in those flies maintained on the naive ear.


EXP. III
RABBITS 5HB5, 5HB6
MEAN PUPAL WEIGHT ± S.D. (mg)
38
34
30
26
22
0
1
2
3
4
5
LARVIPOSITION CYCLE (9 DAY)
NS
NS
NS
*
*
T-TEST * P< 0·05 NS = NOT SIGNIFICANT
△ LEFT EAR (PREVIOUSLY EXPOSED)
● RIGHT EAR (NAIVE)

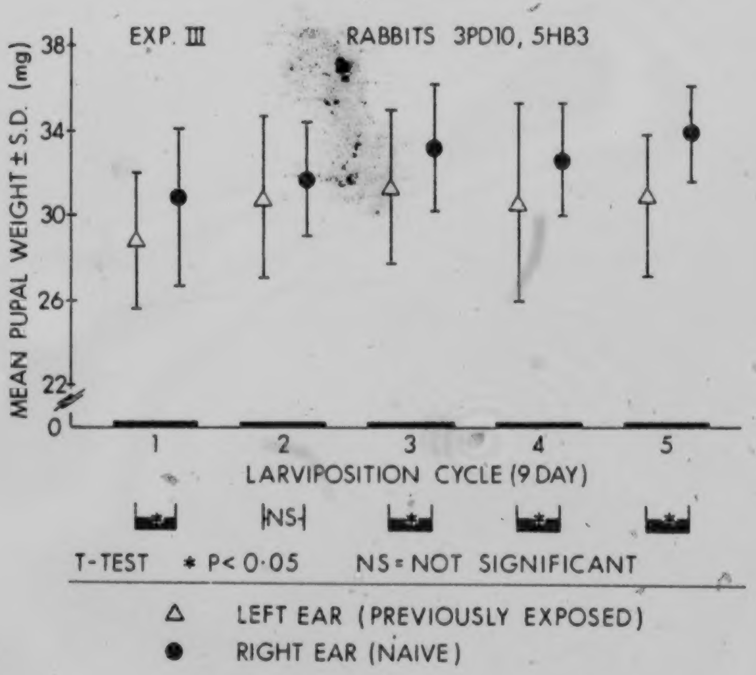

EXP. III
RABBITS 3PD10, 5HB3
MEAN PUPAL WEIGHT ± S.D. (mg)
38
34
30
26
22
0
1
2
3
4
5
LARVIPOSITION CYCLE (9 DAY)
*
NS
*
*
*
T-TEST * P< 0·05 NS = NOT SIGNIFICANT
△ LEFT EAR (PREVIOUSLY EXPOSED)
● RIGHT EAR (NAIVE)

Table 34. ANOVA of pupal weights of flies fed on the naive and previously-exposed ears of rabbits 5HB5 and 5HB6 (the pair of rabbits with the least difference in fly meal weights between the previously-exposed and naive ears).

Source of variation	Sum of squares	DF	Mean square	F
Between ears	3.588	1	3.588	4.253
Residual	6.749	8	0.844	
Total	10.337	9	1.149	

Multiple classification analysis

Grand mean = 32.26 mg

Variable	Status	No. of cycles	Deviation from mean (mg)
Ear on which flies fed:			
Right	naive	5	0.60
Left	previously and presently exposed	5	- 0.60

Table 35. ANOVA of pupal weights of flies fed on the naive and previously-exposed ears of rabbits 3PD10 and 5HB3 (the pair of rabbits with the greatest differences in meal weights between the previously-exposed and naive ears).

Source of variation	Sum of squares	DF	Mean square	F
Between ears	7.939	1	7.939	5.749 *
Residual	11.047	8	1.381	
Total	18.986	9	2.110	

Multiple classification analysis

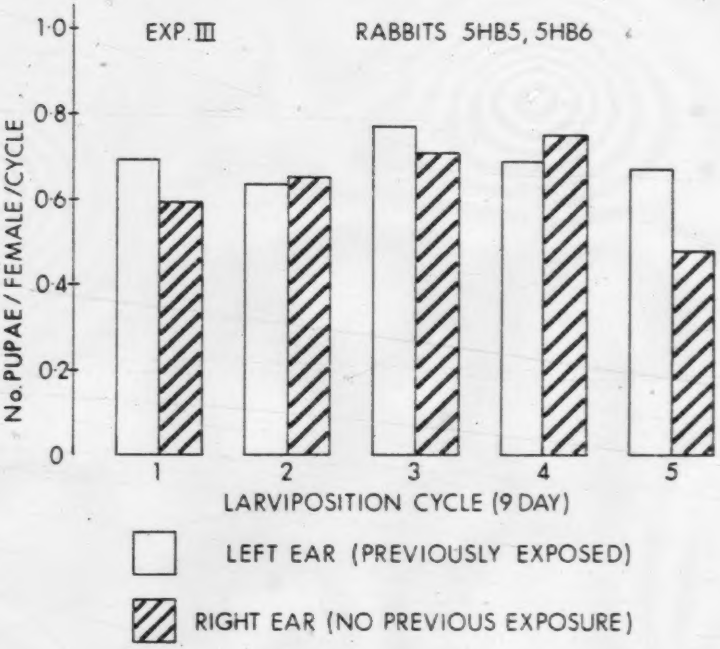
Grand mean = 31.61 mg

Variable	Status	No. of cycles	Deviation from mean (mg)
Ear on which the flies fed:			
Right	naive	5	0.89
Left	previously and presently exposed	5	- 0.89

Level of significance * $P < 0.05$

Analysis includes those pupae less than 20 mg weight.

Figure 60. Female productivity of flies used in Experiment III (local or systemic resistance). In both pair of rabbits, female productivity was not affected when flies were maintained on the previously-exposed ear.


EXP. III
RABBITS 5HB5, 5HB6
No. PUPAE / FEMALE / CYCLE
1·0
0·8
0·6
0·4
0·2
0
1
2
3
4
5
LARVIPOSITION CYCLE (9 DAY)
LEFT EAR (PREVIOUSLY EXPOSED)
RIGHT EAR (NO PREVIOUS EXPOSURE)

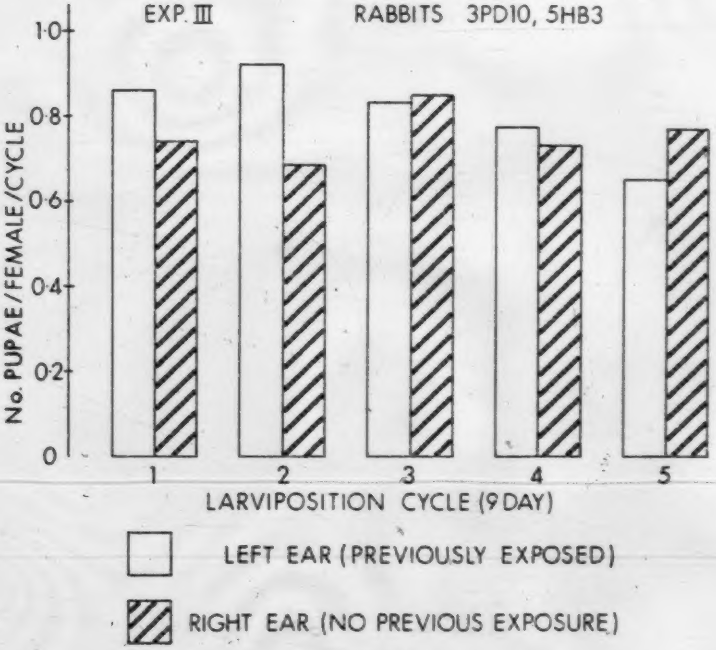

EXP. III
RABBITS 3PD10, 5HB3
No. PUPAE / FEMALE / CYCLE
1·0
0·8
0·6
0·4
0·2
0
1
2
3
4
5
LARVIPOSITION CYCLE (9 DAY)
LEFT EAR (PREVIOUSLY EXPOSED)
RIGHT EAR (NO PREVIOUS EXPOSURE)

Table 36. ANOVA of productivity of female flies maintained on the naive and previously-exposed ears of rabbits 5HB5 and 5HB6 (the rabbits with the least difference in fly meal weights between the previously-exposed and naive ears).

Source of variation	Sum of squares	DF	Mean square	F
Between ears	0.006	1	0.006	2.042
Residual	0.025	8	0.003	
Total	0.032	9	0.004	

Multiple classification analysis

Grand mean = 1.31 Female productivity = $\sqrt{\text{Prod.} + 0.05}$

Variable	Status	No. of cycles	Deviation from mean
Ear on which flies fed:			
Right	naive	5	-0.03
Left	previously and presently exposed	5	0.03

Table 37. ANOVA of productivity of female flies maintained on rabbits 3PD10 and 5HB3 (the pair of rabbits with the greatest difference in fly meal weights between the previously-exposed and naive ears).

Source of variation	Sum of squares	DF	Mean squares	F
Between ears	0.002	1	0.002	1.071
Residual	0.017	8	0.002	
Total	0.019	9	0.002	

Multiple classification analysis

Grand mean - 1.38

Female productivity = $\sqrt{\text{Prod.}} + 0.05$

Variable	Status	No. of cycles	Deviation from mean
Ear on which flies fed:			
Right	naive	5	- 0.02
Left	previously and presently exposed	5	0.02

Analysis includes only those pupae over 20 mg weight.

4.5.3.4 Emergence from pupae

Emergence from pupae was not affected in flies maintained on the previously-exposed ears of either pair of rabbits (Table 38). The sex ratio of the flies which emerged was close to the expected ratio of 1:1 (Table 38).

4.5.4 Experiments IV and V - Effect of naturally produced antibodies

Experiment IV and V will be considered together, since their designs are similar. At the end of Experiment IV, the results could not be attributed to the effect of antibodies alone, since upon examining the haematocrits of naive and previously-exposed rabbits, it was clearly apparent that previously-exposed rabbits had become anemic (Fig. 61A). Experiment V is essentially a replicate of Experiment IV, except that a new group of control rabbits were used, and control rabbits were bled prior to tsetse exposure in an unsuccessful attempt to have the same haematocrit values in naive and previously-exposed rabbits. However, the advantage of higher haematocrit values which had existed in naive rabbits throughout Experiment IV was eliminated (Fig. 61B).

4.5.4.1 Adult survivorship

In Experiment IV, survivorship of female flies fed on the naive ear of the previously-exposed rabbits was the same as the survivorship of flies fed on the naive rabbits (Fig. 62). In Experiment V, survivorship of female flies was significantly lower in flies maintained on the anemic, naive rabbits (Fig. 62). At the end of 60 days, there was a 7% difference in the survivorship between the two populations (Fig. 62).

Table 38. Emergence from pupae collected from flies used in Experiment III (local or systemic resistance). Norm = flies fed on the naive right ear of rabbits. Exp. = flies fed on the previously-exposed left ear of rabbits.

Larviposition cycle	No. pupae produced by flies		No. flies emerged		No. female flies to emerge		Emergence (%)	
(9 day)	Norm	Exp	Norm	Exp	Norm	Exp	Norm	Exp
Flies fed on rabbits 5HB5 and 5HB6 (rabbits with least difference in fly meal weights between previously-exposed and naive ears).								
1	30	36	25	36	13	18	83	100
2	33	33	29	30	18	17	88	91
3	35	40	35	35	16	18	100	100
4	37	35	35	35	23	29	95	100
5	24	35	22	34	11	15	92	88
Total	159	179	146	170	81	87	92	95
Flies fed on rabbits 3PD10 and 5HB3 (rabbits with least difference in fly meal weights between previously exposed and naive ears).								
1	62	67	56	61	31	37	90	91
2	58	72	49	60	30	23	84	83
3	72	66	66	66	29	36	92	100
4	61	59	50	50	21	24	82	85
5	63	49	47	44	27	23	75	90
Total	316	313	268	281	138	193	85	90

Figure 61. Haematocrit levels of the rabbits on which the flies used in Experiments IV and V (effect of naturally produced antibodies) were fed.

A. Haematocrit differential for Experiment IV.

▓ = Haematocrit of naive rabbits
░ = Haematocrit of previously-exposed rabbits

Note that throughout the 10 weeks of the experiment, haematocrit levels in the naive rabbits were well above those of the previously-exposed rabbits. The previously-exposed rabbits received an additional daily tsetse exposure of approximately 300 to 500 flies.

B. Haematocrit differential for Experiment V.

▓ = Haematocrit of naive rabbits (bled anemic)
░ = Haematocrit of previously-exposed rabbits (naturally anemic)

Naive rabbits were bled prior to use in an attempt to reduce differential haematocrit levels which existed in Experiment IV (A.). Although haematocrit levels were seldom equal, no one fly population had the advantage of feeding on rabbits with higher haematocrits over the entire 10 week period.

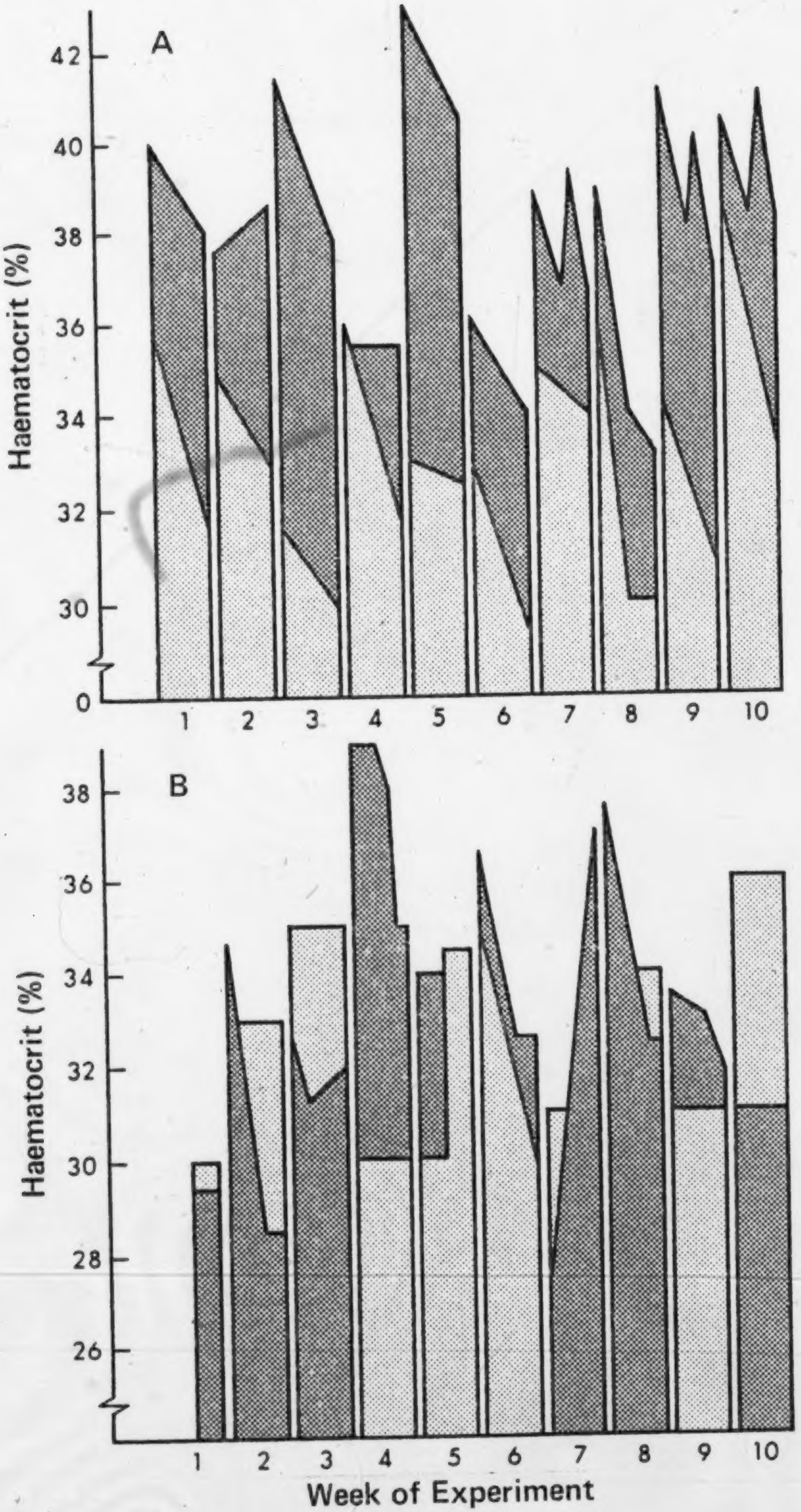
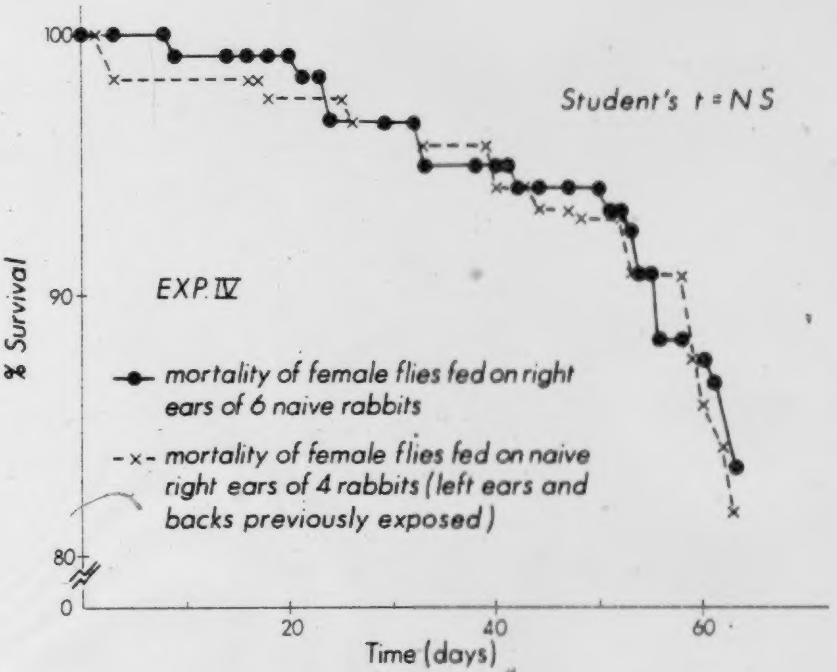
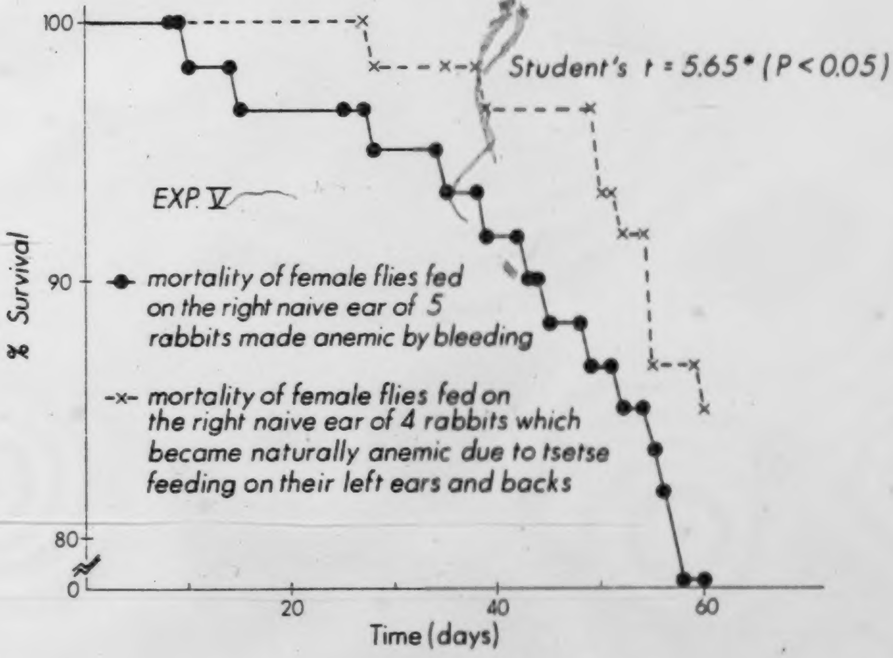

A
B
Haematocrit (%)
42
40
38
36
34
32
30
0
26
28
1
2
3
4
5
6
7
8
9
10
Week of Experiment

Figure 62. Survival curves of flies used in Experiments IV and V (effect of naturally produced antibodies). In Experiment IV, mortality was not affected. In Experiment V, survivorship of flies maintained on the naive rabbits was lower than the survivorship of flies maintained on the naive ear of previously-exposed rabbits.


Student's t = N S
EXP. IV
% Survival
100
90
80
0
20
40
60
Time (days)
–●– mortality of female flies fed on right ears of 6 naive rabbits
-×- mortality of female flies fed on naive right ears of 4 rabbits (left ears and backs previously exposed)


Student's t = 5.65* (P < 0.05)
EXP. V
% Survival
100
90
80
0
20
40
60
Time (days)
–●– mortality of female flies fed on the right naive ear of 5 rabbits made anemic by bleeding
-×- mortality of female flies fed on the right naive ear of 4 rabbits which became naturally anemic due to tsetse feeding on their left ears and backs

4.5.4.2 Pupal weights

In Experiment IV, weights of pupae obtained from flies maintained on the naive ear of previously-exposed rabbits were significantly lighter ($P < 0.05$) in all 5 larviposition cycles than pupae obtained from flies maintained on the naive rabbits (Fig. 63; Appendix D, Table D7). ANOVA of the mean pupal weights obtained was slightly short of being significant (Table 39).

In experiment V, an interesting situation occurred. There was no consistent pattern as to which group of flies produced the heavier pupae (Fig. 63, Table 40; Appendix D, Table D7). Only in the 5th larviposition cycle was the difference in pupal weights significant ($P < 0.05$) between the two groups (flies fed on the naive rabbits produced the heavier pupae). In Experiment IV, 0.6% of the pupae produced by flies maintained on the naive rabbits, and 1.4% of the pupae produced by flies maintained on the naive ear of the previously-exposed rabbits, were less than 20 mg weight (Appendix D, Table D8). In Experiment V, 0.9% of the pupae produced by flies maintained on the naive (bled anemic) rabbits, and 1.3% of the pupae produced by flies maintained on the naive ear of previously-exposed rabbits (naturally anemic due to excessive tsetse feeding), were less than 20 mg weight (Appendix D, Table D8).

4.5.4.3 Female productivity

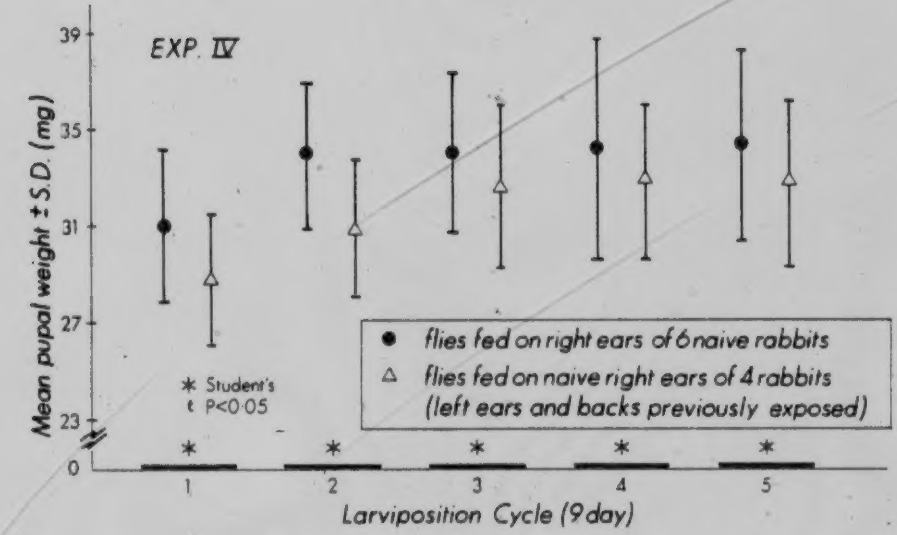
In Experiments IV and V, female productivity was not significantly reduced when flies were maintained on the naive ear of previously-exposed rabbits (Fig. 64, Table 41, 42; Appendix D, Table D8).

4.5.4.4 Emergence from pupae

In Experiments IV and V, emergence from pupae was not significantly reduced in the pupae produced by flies maintained on the naive ear of the

Figure 63. Pupal weights of flies used in Experiments IV and V.

In Experiment IV, mean pupal weights of flies maintained on the naive ear of the previously exposed rabbits were significantly lower than those produced by flies maintained on the naive rabbits. In Experiment V, a replicate of Experiment IV except that naive rabbits were bled so that previously exposed and naive rabbits would have equal haematocrits, no one population produced consistently heavier pupae.


EXP. IV
Mean pupal weight ± S.D. (mg)
39
35
31
27
23
0
1
2
3
4
5
Larviposition Cycle (9 day)
* Student's t P<0·05
* * * * *
● flies fed on right ears of 6 naive rabbits
△ flies fed on naive right ears of 4 rabbits (left ears and backs previously exposed)

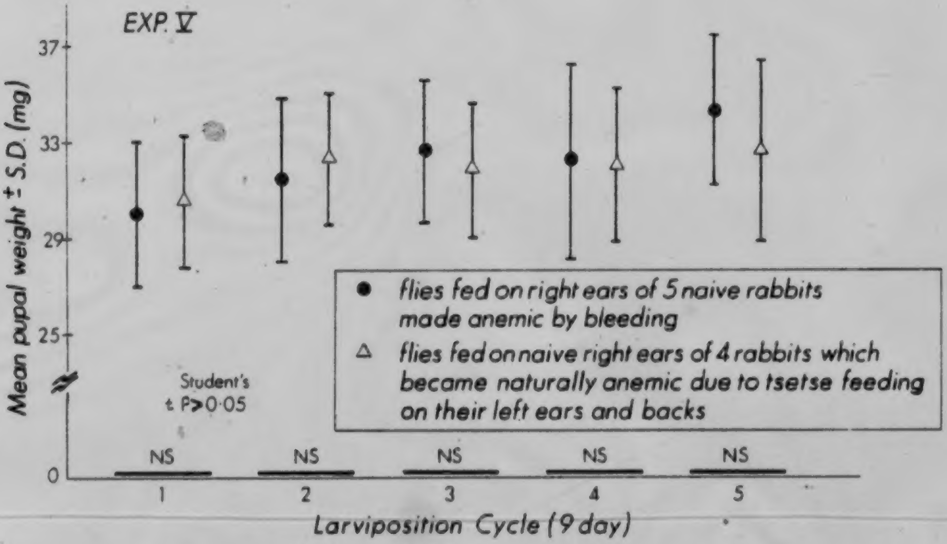

EXP. V
Mean pupal weight ± S.D. (mg)
37
33
29
25
0
1
2
3
4
5
Larviposition Cycle (9 day)
Student's t P>0·05
NS NS NS NS NS
● flies fed on right ears of 5 naive rabbits made anemic by bleeding
△ flies fed on naive right ears of 4 rabbits which became naturally anemic due to tsetse feeding on their left ears and backs

Table 39. ANOVA of pupal weights of flies used in Experiment IV (effect of naturally produced antibodies).

Source of variation	Sum of squares	DF	Mean square	F
Between groups of rabbits	9.025	1	9.025	3.633
Residual	19.874	8	2.484	
Total	28.899	9	3.211	

Multiple classification analysis

Grand mean = 32.52 mg

Variable	Status	No. of cycles	Deviation from mean (mg)
Rabbit group used to feed flies:			
6 control	naive	5	0.95
4 experimental	previously and presently exposed	5	- 0.95

Analysis includes those pupae less than 20 mg weight.

Table 40. ANOVA of pupal weights of flies used in Experiment V (effect of naturally produced antibodies).

Source of variation	Sum of squares	DF	Mean square	F
Between groups of rabbits	0.130	1	0.130	0.083
Residual	12.556	8	1.570	
Total	12.686	9	1.410	

Multiple classification analysis

Grand mean = 31.97 mg

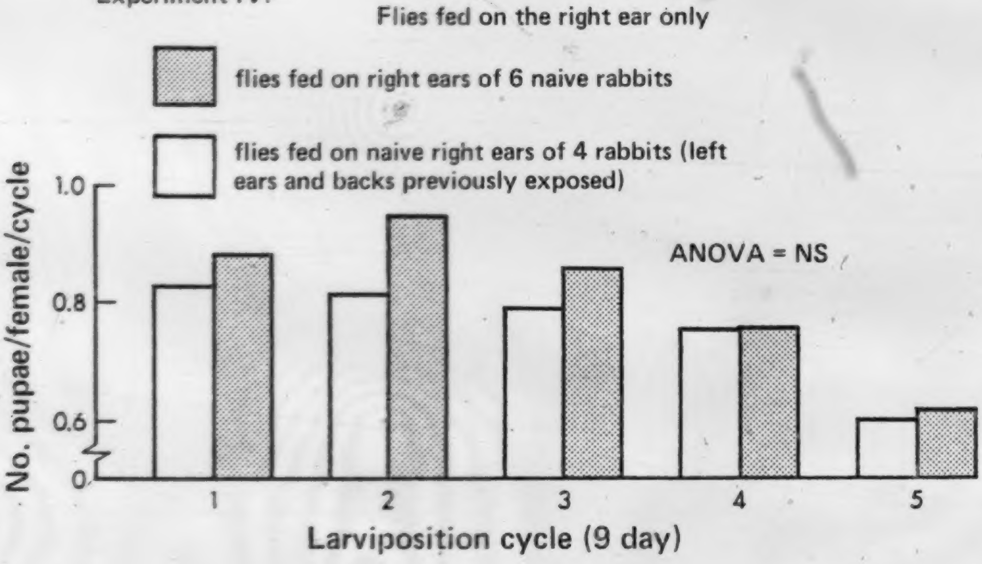
Variable	Status	No. of cycle	Deviation from mean (mg)
Rabbit group used to feed flies			
5 control[a]	naive	5	0.11
4 experimental	previously and presently-exposed	5	- 0.11

Analysis includes pupae less than 20 mg weight.

[a] Control rabbits were bled so that haematocrit values would be equal to those of experimental rabbits, made anemic by excessive tsetse feeding.

Figure 64. Female productivity of the flies used in Experiments IV and V (effect of naturally produced antibodies). In both experiments, productivity was not reduced in those flies maintained on the naive ear of the previously-exposed rabbits.

Experiment IV:



Experiment V:

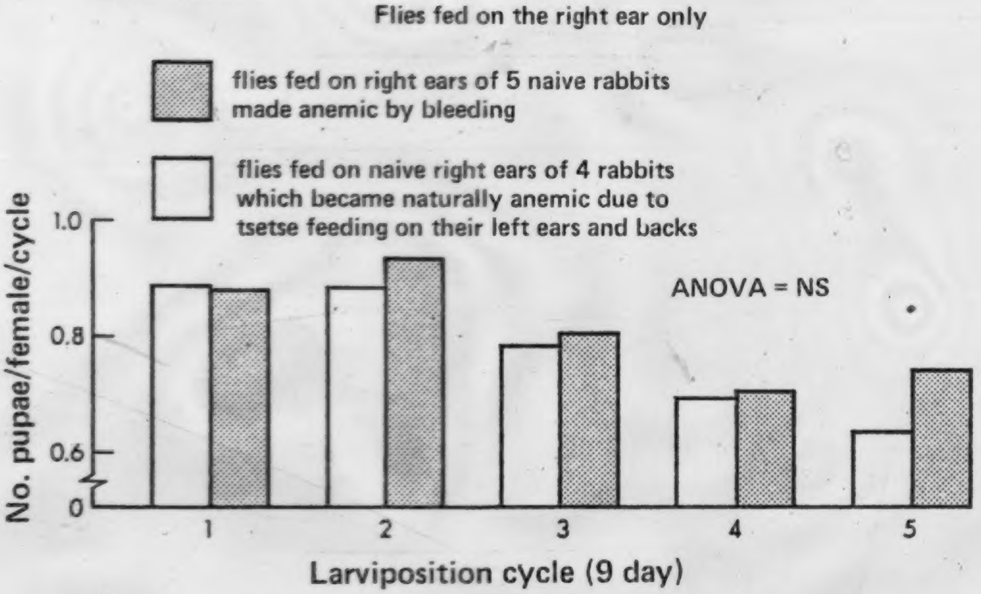


Table 41. ANOVA of productivity of female flies used in Experiment IV.

Source of variation	Sum of squares	DF	Mean square	F
Between groups of rabbits	0.002	1	0.002	0.542
Residual	0.033	8	0.004	
Total	0.036	9	0.004	

Multiple classification analysis

Grand mean = 1.38 Female productivity = $\sqrt{\text{Prod. + 0.05}}$

Variable	Status	No. of cycles	Deviation from mean
Groups of rabbits used to feed flies:			
6 control	naive	5	0.02
4 experimental	previously and presently exposed	5	- 0.02

Analysis includes only those pupae over 20 mg weight.

Table 42. ANOVA of productivity of female flies used in Experiment V.

Source of variation	Sum of squares	DF	Mean square	F
Between groups of rabbits	0.001	1	0.001	0.312
Residual	0.028	8	0.004	
Total	0.029	9	0.003	

Multiple classification analysis

Grand mean = 1.39 Female productivity = $\sqrt{\text{Prod.} + 0.05}$

Variable	Status	No. of cycles	Deviation from mean
Groups of rabbits used to feed flies:			
5 control[a]	naive	5	0.01
4 experimental	previously and presently exposed	5	- 0.01

[a] Control rabbits were bled so that haematocrit values would be equivalent to experimental rabbits which were made anemic by excessive tsetse feeding.

Analysis includes only those pupae over 20 mg weight.

previously-exposed rabbits (Table 43). Similarly, the sex ratio of the flies was not affected (Table 43).

4.6 Discussion

In the first two experiments, designed to examine those aspects of the life cycle of the tsetse affected by host-resistance, the results had similar trends. Female survivorship, female productivity, and pupal weights were affected in those flies maintained on the previously-exposed rabbit. Male survivorship and emergence from pupae were not. The use of a control rabbit of the same litter as the previously-exposed rabbit eliminated the possibility that the results may be attributed to an age differential. A definite resistance, detrimental to the tsetse, develops in those rabbits previously-exposed. There are at least 3 possible explanations for an effect on the female, but not the male tsetse. First, a specific mechanism, possibly immunological in nature, may be detrimental to the female tsetse but not to the male. Most of the exposure required to make a rabbit 'previously-exposed' was from female flies (most of the colony is productive females). It is possible that an immunological mechanism, perhaps a detrimental antibody, is formed in host-rabbits toward a salivary component found only in the female tsetse. This possibility is reduced by the fact that immunoelectrophoretic patterns of both male and female salivary glands were similar (see Section 3.4.2.1). However, a detrimental non-precipitating antibody may also be involved.

Second, a mechanism restricting meal size, perhaps skin thickening or vasoconstriction, may be involved. A reduction in the meal size taken by the male flies may not significantly interfere with metabolic processes. However, the female tsetse, being larviparous, donates a large portion of

Table 43. Emergence from pupae collected from flies used in Experiments IV and V (effects of naturally produced antibodies).

Experiment IV: Norm = flies fed on 6 naive rabbits

Ab = flies fed on the naive ear of 4 previously-exposed rabbits.

Larviposition cycle	No. pupae produced		No. flies emerged		No. female flies to emerge		Emergence (%)	
	Norm	Ab	Norm	Ab	Norm	Ab	Norm	Ab
1	105	97	96	76	42	37	91	78
2	109	94	95	85	46	46	87	90
3	97	89	92	81	43	38	95	91
4	84	83	74	72	36	29	88	87
5	65	64	54	55	30	34	83	86
Total	460	427	411	369	197	184	89	86

Experiment V: HC = flies fed on 5 naive rabbits which were bled until haematocrits were similar to those of Ax rabbits.

Ax = flies fed on the naive ear of 4 previously-exposed rabbits made anemic by excessive tsetse feeding.

Larviposition cycle	No. pupae produced		No. flies emerged		No. female flies to emerge		Emergence (%)	
	Norm	Ab	Norm	Ab	Norm	Ab	Norm	Ab
1	53	53	45	44	21	21	85	83
2	54	54	44	45	26	26	81	83
3	46	46	28	35	17	16	61	76
4	39	44	27	32	12	17	69	73
5	36	34	27	22	13	13	75	65
Total	228	231	171	178	89	93	75	77

her blood meal to the developing larva. A reduction in her meal size would mean less nutrient is available for the female, possibly resulting in a stress which might increase mortality and decrease productivity. At the same time, less nutrient would be available for the developing larva, resulting in lighter weight pupae. In correlation with this possibility, Bursell(1965) suggests that because of the energy expenditure of the tsetse required to convert waste material to uric acid, considerably less than 50% of the blood meal becomes available as a source of metabolic energy. Langley and Pimley (1975) state that 'since the fly must utilize a portion of the remainder (the other 50%) in order to satisfy her own metabolic requirements, and at least 30% of the total protein ingested between one larviposition and the next will appear in the fully grown third-instar larva, it is a simple deduction that even a 10% shortfall in nutrient intake by the female fly will result in production of significantly undersized offspring.' The reason for an increase in female mortality but not male mortality is further complicated by the results of fly meal experiments (see Section 3.4.1). Fly meal experiments demonstrated that there was no significant difference in the meal sizes taken from previously-exposed and naive rabbits (or portions of rabbits). These results are difficult to interpret since the size of the blood meal varies widely. However, it was further demonstrated that male tsetses probe more frequently and exert more effort to obtain a blood meal than females (Section 3.4.3.1). Although the extra effort exerted by the male tsetse may give it a better chance to obtain a blood meal, it also increases the energy cost per blood meal. Another point to consider is that the energy exerted in obtaining a blood meal under the unnatural confinement to cages may actually account for very little of the total energy budget of the fly. With respect to the mechanism, it seems more probable that arteriolar vasoconstriction is involved than skin thickening. In a review, Nelson et al. (1977) reported that 'skin thickness could not be correlated with innate resistance of cattle to Boophilus microplus (Riek, 1962), and seems to have no bearing on resistance to lice or sheep keds (Nelson, unpubl.).' Not only was arteriolar vasoconstriction observed in those rabbits exposed to 1200 to 1500 flies over a 4 hour period, but it also plays a part in resistance in the ked-sheep and louse-mouse systems (see review by Nelson et al., 1977 and Section 3.1).

A third possible explanation for the results obtained in Experiments I and II is that meal sizes are not affected, but that the nutrient level of the blood meal is reduced. In Experiment IV, it was demonstrated that rabbits exposed to both experimental and colony flies became anemic, whereas rabbits receiving only exposure to experimental flies did not. Once the factor of anemia had been eliminated (Experiment V), there was no longer a difference between weights of pupae produced by flies maintained on the naive ear of previously-exposed rabbits and the weights of pupae produced by flies maintained on the ears of naive rabbits. These results indicated that anemia and not antibodies were interfering with tsetse pupal weights. Similarly, anemia, or lack of some other component in the blood meals taken by flies feeding on the previously-exposed rabbits in Experiments I and II may account for the decrease in pupal weights. It does not however, account for lower female survivorship unless the lack of some nutrient in the blood meal is also detrimental to the female, similar to the effect of a reduced meal size discussed earlier. Unfortunately, haematocrit levels of the rabbits used in Experiments I and II were not examined, leaving the possible influence of anemia on pupal weights in these experiments uncertain. Other possibilities such as the effects of serum inhibitor levels remain unknown, since at the present time, there are very few known physiological or biochemical differences (excluding the reproductive system) distinguishing the male from the female tsetse (pers. commun. with R. H. Gooding).

In experiment III (local versus systemic resistance), different levels of resistance were acquired between the two pair of rabbits. In the pair of rabbits with the least differences in fly meal weights between ears, adult survivorship at the end of 63 days was not affected although pupal weights were reduced. In the pair of rabbits with the greatest difference in meal weights between ears, survivorship and pupal weights were lower in those flies maintained on the previously-exposed ear. These results suggest that there is a difference in the level of resistance between the two pair of rabbits. Results further suggest that pupal weights are more sensitive indicators of resistance than mortality. Female productivity and emergence from pupae were least affected. Even though the differences in the meal weights between ears of either pair of rabbits were never significant, there was still a difference in the meal weights obtained by flies which were maintained on the previously-exposed ears. This further substantiates my earlier conclusion that fly meal weights are a variable and perhaps unsuitable factor for determining levels of resistance. Since there was a difference in the response of those flies maintained on the previously-exposed ears, resistance is described as local. Local resistance has also been reported in mice infested with Polyplax serrata (Bell et al., 1966), in sheep infested with Melophagus ovinus (Nelson and Bainborough, 1963; review by Nelson et al., 1977), and suggested in cattle infested with Haematopinus eurysternus (see review by Nelson et al., 1977).

Results of Experiments IV and V (designed to determine if naturally produced antibodies are detrimental to the tsetse) indicate no fly responses could be attributed to the role of host antibodies. Although pupal weights of flies maintained on the naive ear of previously-exposed rabbits (those with high antibody titres and presumably no local resistance) in Experiment IV were lower than controls (no antibodies, no local resistance), with the elimination of anemia (caused by excessive tsetse feeding), this difference no longer existed. These results do not agree with the results obtained with flies maintained on hosts immunized with fly tissues (Alger and Cabrera, 1972; Sutherland and Ewen, 1974; Schlein and Lewis, 1976).

At present, resistance in host rabbits has been shown as local. No detrimental effects could be attributed to the presence of naturally produced circulating antibodies. The mechanisms involved with host resistance remain uncertain, although arteriolar vasoconstriction may play a substantial role.

One interesting point is the effect of host anemia on tsetse pupal weights. It is well known that ectoparasites thrive best on hosts in poor physiological condition, primarily because levels of host-resistance are lower (see review by Nelson et al., 1975, 1977). These are the same hosts which become heavily infested with ectoparasites and consequently become anemic. Since host anemia affects the parasite, this suggests that the parasite must choose a host, or create a host whose physiology is sufficiently altered to reduce the host's resistance to the parasite, but not so much altered (as in excessive anemia) as to create conditions which are detrimental to the ectoparasite or its progeny.

5.0 General summary

An artificial situation, produced using a group of Flemish Giant x French Lop-eared rabbits and a colony of uninfected *Glossina morsitans morsitans* (Westw.), was used to examine host biting-fly interactions. Three aspects were examined. Tests were performed to clarify the mechanism of action of the tsetse salivary anticoagulant and to further investigate the effects of salivary gland secretions on haemostasis. Some physiological responses in host-rabbits, particularly haematological and serological reactions, were examined throughout various levels of tsetse exposure. Naturally acquired host-resistance, developed through previous tsetse exposure, was examined for its detrimental effect on various biological parameters of the tsetse.

The anticoagulant in the salivary gland homogenate was demonstrated to be an antithrombin of low molecular weight, distinct from both heparin and hirudin. It acts on at least 2 sites, inhibiting both thrombin's proteolytic and esterolytic activity. As thrombin-induced platelet aggregation was also completely inhibited and thrombin binds with platelets at a site different from its proteolytic site (Cooper et al., 1976), a third site may also be involved. The action of the antithrombin was immediate and it remained stable at 100°C for 60 minutes and for at least one month below +4°C. Salivary gland homogenates contained no haemolytic or fibrinolytic activity but they inhibited platelet aggregation induced by thrombin, ADP, acid collagen, and adrenaline. Ristocetin-induced platelet aggregation was not affected. The action of the salivary gland homogenate on platelet aggregation is suggested to be a result of the antithrombin in SGS. Salivary gland homogenate did not affect platelet adherence to collagen.

Host-rabbits exposed to 250-500 flies per day, 2 to 3 days a week, did not show significant differences in haematocrit levels, weights, red and white blood cell counts, or whole blood clotting times from rabbits receiving no exposure. The same level of exposure 6 days a week resulted in sharp decreases in haematocrit levels, and in some cases changes in weights. Haematocrit levels correlated negatively with tsetse exposure. Sharp increases in weights of some female rabbits suggests that salivary toxins may affect host-metabolism. Weights and haematocrit levels of most rabbits exposed to 1200 to 1500 flies on a single occasion were not affected. However, following exposure, pronounced arteriolar vasoconstriction, believed to play a role in host-resistance, was observed. Naturally produced precipitating antibodies against salivary gland homogenate were detected in rabbits previously-exposed to tsetses. As many as 7 antigen-antibody precipitation arcs were observed using immunoelectrophoresis, with development of some bands being a function of time, under a constant level of tsetse exposure. Passive haemagglutination titres to naturally injected saliva were measured in the sera of rabbits and found to correlate, to some extent, with the level of tsetse exposure. Fly blood-meal weights, used to measure local resistance, proved unsatisfactory for this purpose. Female teneral flies probed less frequently and took larger blood meals than males. The backs of rabbits proved less favourable for obtaining a blood meal than either a previously-exposed or naive ear. The number of times a fly probed correlated negatively with meal size.

Five experiments, each 2 months in length, demonstrated that naturally developed host-resistance to previous tsetse exposure affects biological parameters of the tsetse. In two experiments, designed to determine which biological parameters are affected by host-resistance, female survivorship, female productivity and pupal weights were lower in those flies maintained on previously-exposed rabbits. Male mortality and emergence of both sexes from pupae were not affected. A third experiment demonstrated that resistance is local, that pupal weight is a more sensitive indication of host-resistance than is adult survivorship and that rabbits develop different levels of resistance. In the last two experiments, designed to determine if naturally produced antibodies to tsetse salivary gland homogenates are detrimental to the tsetse, none of the biological parameters were affected by the presence of circulating antibodies in the host-rabbits. However, anemia, caused by excessive tsetse feeding, reduced pupal weights.

Naturally produced precipitating antibodies against tsetse salivary gland homogenate did not affect the anticoagulant activity of the salivary gland homogenate and could not be demonstrated as causing detrimental effects on various biological parameters of the tsetse. The role of these antibodies in host-ectoparasite interaction or host-resistance remains uncertain.

Although the anticoagulant of the salivary glands was demonstrated as being both potent and stable, excessive exposure of rabbits to tsetse failed to affect *in vivo* clotting times. Even though 7 precipitin arcs formed when salivary gland homogenate was electrophoresed and incubated with rabbit antisera, the Sephadex fractions of the salivary glands which contained the maximum anticoagulant activity produced no precipitin reaction when incubated with antisera.

A ratio of the average blood meal weight over the average number of times a teneral tsetse probes to obtain a blood meal was used as a measure of feeding efficiency. Female tsetses were more efficient at obtaining a blood meal than males, however feeding flies on previously-exposed rabbits for a period of 2 months resulted in higher mortality rates in females than in males. These results may be due to the greater nutrient demands of pregnant females. Levels of excessive tsetse exposure resulted in host-anemia. Flies maintained on such rabbits had lower pupal weights, indicating that the choice of host is important.

5.1 Applicability of results to tsetse husbandry

The importance of this research to tsetse husbandry has been outlined in Section 1.3. The results of Chapter 4 demonstrated that naturally acquired resistance in host-rabbits can reduce tsetse pupal weights, decrease adult survivorship, and may influence female productivity. Pupal weights were most sensitive to resistance, followed by mortality. Female survivorship was affected more than male survivorship. In no case was emergence from pupae affected. Interchanging either previously-exposed or naive rabbits once a week on a 4 or 6 week cycle produced pupae 1.3 to 5.6 mg (3.8% to 16.4%) heavier than flies maintained on one rabbit previously exposed daily for a period of 2 months. Detrimental effects on the parameters of the tsetse measured were eliminated through interchanging previously-exposed rabbits once a week on a 4 week cycle. Through such interchange, flies maintained on previously-exposed or naive rabbits produced pupae with mean weights of 32 to 34 mg, significantly heavier than pupae produced by flies maintained on artificial membranes or flies maintained on rabbits or other hosts from other colonies (Willett, 1955; Foster, 1957; Langley and Pimley, 1975; Mews et al., 1976; review by Laird, 1977). Rabbits used heavily in the past, and then used to feed flies 6 days a week, produced the greatest resistance to the tsetse. Resistance was demonstrated as local rather than systemic, indicating that areas of previous exposure are important. It is believed that arteriolar vasoconstriction of blood vessels in the ears of the rabbits plays a role in resistance. Fly exposure should therefore be distributed over both ears and back or limited in a specific area. Flies were also demonstrated to take significantly lighter blood meals from the back than from either a naive or a previously-exposed ear. Since small reductions in the meal size may result in significantly undersized offspring (Langley and Pimley, 1975), it would be advisable to limit tsetse exposure to the rabbit's ears. Rabbits were able to withstand tsetse exposure 6 days a week for over 20 weeks, or one exposure to 1200 to 1500 flies in 4 hours, without serious effects. Rabbits used over long periods became anemic, although such a condition never resulted in death. Experiments indicated that tsetse pupal weights are reduced when flies are maintained on rabbits made anemic by excessive tsetse feeding. Hosts should therefore be used for limited periods of time to reduce the possibility of the host becoming anemic or developing resistance. The cross of rabbit used to maintain the tsetses was adequate. Over the two years of this research, and using 24 to 30 rabbits, only in 1 was there a noticable thickening of skin on the ears.

5.2 Implications of results for studies of ectoparasites in general

Nelson et al. (1977) reported that only twice have naturally produced precipitating antibodies been demonstrated in hosts exposed to arthropods other than the acarina. My experiments with rabbit sera and tsetse salivary gland homogenates now represent the third such report. More antibody-antigen precipitin arcs were observed in my experiments than in rabbits and guinea pigs exposed to *Aedes aegypti* (Wilson and Clements, 1965; 3 arcs were observed) or in rabbits exposed to Rhodnius prolixus (Fox and Bayona, 1968; 3 arcs were observed), probably, for one reason, because I used immunoelectrophoretic techniques rather than simple gel diffusion. Since there are at least seven antigens in tsetse salivary gland homogenate, and since all of the rabbits previously-exposed to tsetses that were examined had antibodies in their sera, the tsetse-rabbit model appears superior, to other, previously reported , systems, for further immunological investigation. At present, only one arthropod antigen (from the tick, Boophilus microplus) has been isolated and partially characterized (Willadsen and Williams, 1976).

Serological responses (antibody titres) in hosts exposed to ectoparasites other than the acarina, have apparently been examined only once, and unfortunately the results have not been published (Robertson and Nelson, unpubl., from review by Nelson et al., 1977). Comparing the titres of rabbits exposed to tsetses with published reports on titres in hosts exposed to ticks and Hypoderma sp. (see Section 3.1), the tsetse-rabbit model has apparently produced the most successful application (highest titres) of this technique to host-ectoparasite interactions. Furthermore, unlike some studies with ticks and cattle grubs (Boulard and Weintraub, 1973; Brossard, 1976), passive haemagglutination titres in rabbits exposed to tsetses are responses to naturally injected antigens (in this model, antigens injected during feeding). It has been demonstrated that artificial hosts such as rabbits infested with Hypoderma sp. respond serologically different from natural hosts (Boulard and Weintraub, 1973, Brossard, 1976). Such reports have an important bearing on my results, since I used an artificial combination of host and insect. Biting-flies however, unlike ticks (Brossard, 1976) and cattle grubs (Boulard and

Weintraub, 1973), are intermittent feeders. Valid comparison is therefore difficult. My research has examined a number of aspects of host biting-fly interaction which have been largely neglected. Regardless of whether or not some of these results obtained using this artificial combination of host and parasite differ from natural situations, they will still serve as a guideline representing interaction under controlled conditions. Biting flies are usually not host-specific. One would expect that different hosts will respond differently to biting fly attack, depending upon the species of fly, the host's previous exposure, the extent of the exposure, and the physiology of the host.

In review, Nelson et al. (1977) state that the highly reactive host exerts a great selective pressure on the ectoparasite, to modify its antigenic properties and reduce antigenic disparity between host and parasite. In conjunction with this statement, in my experiments the most important component to the salivary glands, the anticoagulant, was not antigenic. These results may be due to the modification of the anticoagulant through a long association as a parasite. Nelson et al. (1977) further state that few authors have associated the development of antibody with the development of resistance. The present study followed this pattern; naturally produced precipitating antibodies against tsetse saliva did not affect the anticoagulant activity of the salivary glands, and could not be demonstrated to be detrimental to the tsetse. Research with the tsetse-rabbit model therefore, has not only produced some unique results, but also strengthened a number of suggestions already proposed.

In contrast to reports that resistance in hosts exposed to acarina is systemic, and in agreement with reports that resistance in hosts exposed to ectoparasites, other than the acarina, is local (louse-mouse and ked-sheep systems; see Section 3.1), resistance in host-rabbits exposed to tsetses was demonstrated as local.

My experiments with tsetse salivary gland homogenate represent the most complete haematological study of its kind directed toward arthropods. Although I am not a haematologist, cooperation of Dr. Mant and members of his staff made this study possible. Hopefully the time and space used to explain the principles of the tests will aid other non-haematologists in understanding and performing similar studies, and to bridge the gap between haematology and medical-entomology. A point of major significance is that researchers should not be reluctant to study related fields outside their own areas of specialization. In this study for example, choosing to examine 3 aspects of this model has probably provided a more integrative understanding of host biting-fly interaction than detailed study of one aspect alone. The complex physiological interactions of the host-parasite interphase represent a challenge to the best of minds. Medical-entomologists are fortunate to be working with an applied field of medicine in as much as progress in medical research over the last 20 years has greatly expanded our scope for studying and understanding host-ectoparasite interactions. Their progress is our advantage, we must use it and apply it.

5.3 Suggestions for further research

1. Determine the functions of the high molecular weight antigenic fractions of the tsetse saliva, and what possible roles they may have in host-ectoparasite interaction.
2. Determine if the naturally produced antibodies to tsetse saliva have detrimental effects on the biology of the trypanosomes.
3. Use the immunoelectrophoretic technique and sera from rabbits containing antibodies to *G. morsitans morsitans* as a taxonomic tool for comparing homologous salivary gland components in other species of *Glossina*, *Stomoxys*, and other muscids.
4. Determine if there is a non-precipitating immunological reaction between the anticoagulant in the saliva and host blood or tissues.
5. Determine if a substantial amount of saliva injected into the host is excreted by the kidneys, as hirudin is (Markwardt, 1958), within a short time after intravenous injection. Rabbit urine could be concentrated and examined for antigenic properties with rabbit antisera, and for anticoagulant activity.
6. Determine more closely the relationship between tsetse saliva and blood platelets in light of the theories put forth by Galun and Rice (1971) and Galun (1975). Immunological reactions have been known to induce platelet aggregation (reviews by Mustard and Packham, 1970, 1975). It might prove worthwhile to determine if platelet aggregation can be induced by mixing tsetse salivary gland solution with platelet-rich plasma from a rabbit with high antibody titres. If platelet aggregation can be induced in this way, it may have some significance in the haematogustatory role of the platelet and on host specificity. A host species selected by a host-specific ectoparasite

will receive exposure from that ectoparasite and consequently immunological responses from its bite. Other ectoparasites of the same species feeding on the previously-exposed host may obtain the stimuli to feed through interaction of saliva with host blood. An immunological reaction between host antibody and ectoparasite saliva may induce the platelets to release among other components, adenosine phosphates, which are known to induce feeding. Ectoparasites which are not host specific will not require such a mechanism.

7. Determine if antibody titres correlate with resistance. Although this was originally planned for this study, interchanging hosts, and titres beyond the range of the test in some cases made comparison impossible.
8. Determine the fate of the naturally produced antibodies to tsetse saliva when ingested by the fly in a blood meal. With the use of immunofluorescent techniques this would be quite feasible.
9. Since arteriolar vasoconstriction decreases blood flow, and blood flow correlates with rabbit ear surface temperature (heat release), it would be interesting to determine if it is feasible to measure surface temperature of the rabbit ear as a means of measuring resistance.
10. Use artificial membrane feeding and blood with antibodies against tsetse saliva from rabbits to substantiate results that antibodies are not detrimental to the tsetse. If, in such an experiment there were detrimental effects, they would be the result of a humoral component. Modifying such an experiment, one could also determine if serum inhibitor levels play a role in host-resistance.
11. Host-blood chemistry has been largely neglected other than in two papers

on cattle response to Boophilus microplus (O'Kelly and Seifert, 1970; O'Kelly et al., 1971). Studies comparable with these could easily be achieved using sera from exposed hosts and a Technicon analyzer, presently used in hospital blood chemistry laboratories. With this machine, at least 12 serum component levels can be measured. I had planned to pursue this possibility with sera samples obtained from rabbits exposed to tsetses, however time did not permit.

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Appendix A

APPENDIX OF CHAPTER 1

Figure A1. Tsetse life cycle in relation to colony maintenance.

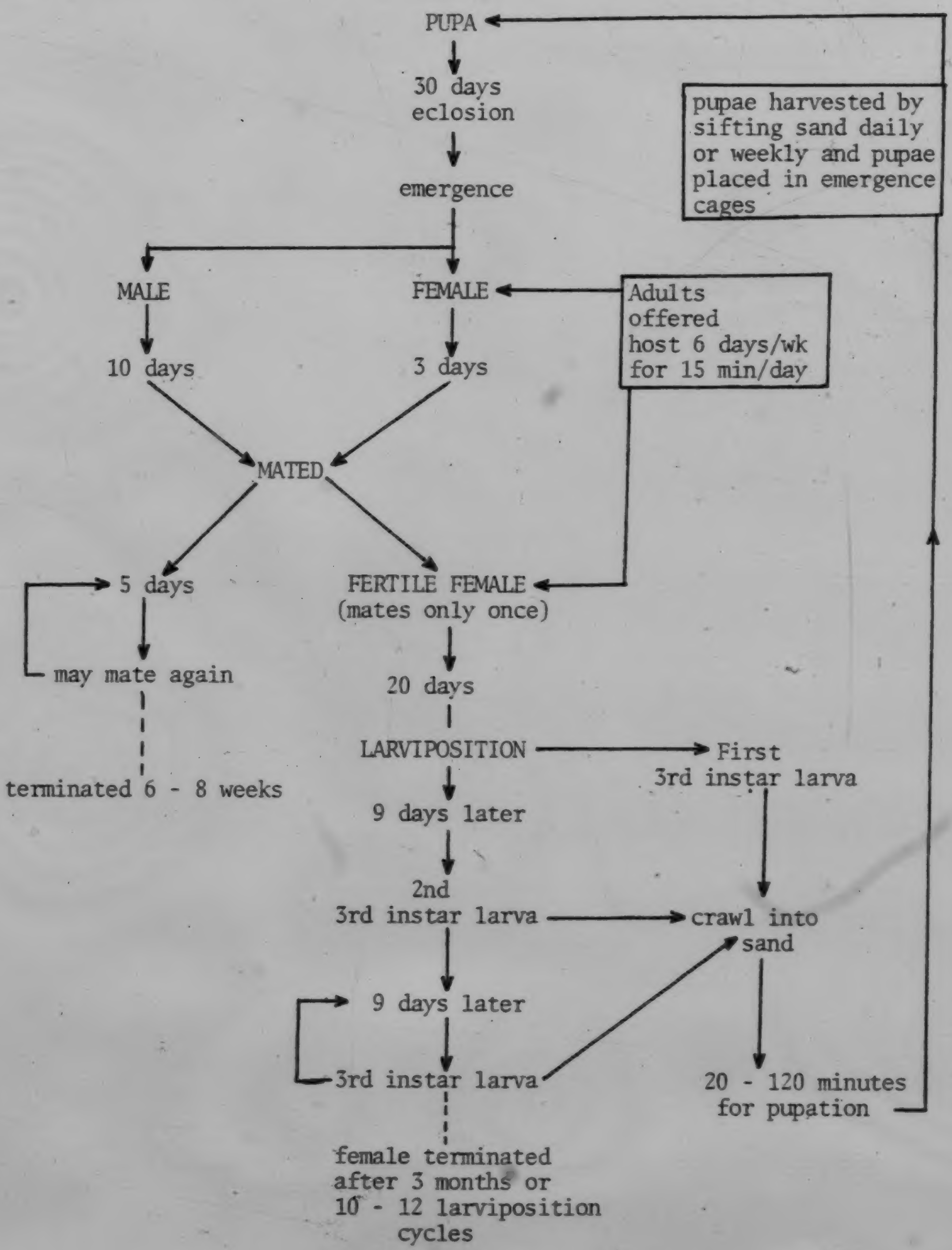
PUPA
30 days
eclosion
emergence
pupae harvested by sifting sand daily or weekly and pupae placed in emergence cages
MALE
FEMALE
Adults offered host 6 days/wk for 15 min/day
10 days
3 days
MATED
5 days
may mate again
terminated 6 - 8 weeks
FERTILE FEMALE (mates only once)
20 days
LARVIPOSITION
First 3rd instar larva
9 days later
2nd 3rd instar larva
crawl into sand
9 days later
3rd instar larva
20 - 120 minutes for pupation
female terminated after 3 months or 10 - 12 larviposition cycles

Table A1. Composition of rabbit diet and estimated weekly nutritional intake. Diet: Master Baby rabbit pellets (Maple Leaf Mills, Edmonton).

Component	Percentage of diet	Estimated weekly[a] consumption
crude protein	(minimum) 18.0	100.7 gm
crude fat	(minimum) 2.5	13.9 gm
crude fibre	(maximum) 12.0	67.1 gm
salt	0.5	2.8 gm
calcium	1.3	7.3 gm
phosphorus	0.75	4.2 gm
vitamin A	(minimum) 5000 IU/lb	6166 IU/week
vitamin D	(minimum) 880 IU/lb	1085 IU/week
vitamin E	(minimum) 2.5 IU/lb	3.8 IU/week

[a] Weekly consumption estimated from a weighted measure of 100 gm of food per day or 700 gm per week. A value of 560 gm was used for nutritional estimates based on an estimated 20% food loss by spillage.

Appendix B

APPENDIX OF CHAPTER 2

Table B1. ANOVA results of platelet aggregation maxima induced by acid collagen and adrenalin .

	Cell Means								
Concentration[a]	Acid collagen			1^{o} Adrenalin			2^{o} Adrenalin		
	NaCl	SGS	$\bar{X}$	NaCl	SGS	$\bar{X}$	NaCl	SGS	$\bar{X}$
1	92.0	58.1	75.1	14.3	15.6	14.9	83.3	81.0	82.4
2	92.5	47.0	69.8	8.1	9.6	8.9	85.8	66.8	76.3
3	76.0	25.6	50.8	4.1	5.0	4.6	85.3	69.5	77.4
4	30.0	9.2	19.6	3.8	3.6	3.8	72.7	17.0	44.8
5	13.9	3.8	8.9	2.1	2.5	2.3	0.0	8.3	4.2
$\bar{X}$	60.9	28.8		6.5	7.3		65.5	48.5	

Source of variation	DF	MS	F	MS	F	MS	F
Trts	1	7744.1	43.4 ***	10.0	0.39	2167.5	12.5 **
Conc'n	4	5250.3	29.5 ***	366.6	14.4 ***	6564.0	57.8 ***
Trts x conc'n	4	421.7	2.4	1.7	0.067	878.5	5.0 **
Error + replicates	20	178.3		25.5		173.9	
Total	29						

* = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.001$

[a] No. representing concentration is arbitrary. Lowest number represents highest concentration. For further details, see methods.

Table B2. ANOVA results of platelet aggregation slopes induced by acid collagen and adrenalin .

	Cell Means								
Concentration[a]	Acid collagen			1^o Adrenalin			2^o Adrenalin		
	NaCl	SGS	$\bar{X}$	NaCl	SGS	$\bar{X}$	NaCl	SGS	$\bar{X}$
1	86.3	50.0	68.2	17.0	21.6	19.3	38.7	43.8	41.3
2	94.0	46.3	70.2	9.0	12.1	10.6	34.6	28.1	31.4
3	58.1	29.9	44.0	4.0	5.6	4.8	35.2	19.4	27.3
4	14.4	5.0	9.7	3.8	1.6	2.7	22.1	5.6	13.8
5	11.2	1.2	6.2	1.4	1.8	1.6	0.0	1.6	0.8
$\bar{X}$	52.8	26.4		7.0	8.5		26.1	19.7	

Source of variation	DF	MS	F	MS	F	MS	F
Trts	1	5191.6	50.9 ***	38.7	1.3	398.5	4.9 *
Conc'n	4	5670.1	55.6 ***	745.9	24.5 ***	1500.5	24.0 ***
Trts x conc'n	4	416.7	4.1 *	23.8	0.8	144.6	2.3
Error + replicates	20	102.0		30.5		62.7	
Total	29						

* = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.001$

[a] No. representing concentration is arbitrary. Lowest number represents highest concentration. For further details, see methods.

Table B3. ANOVA results of platelet aggregation maxima induced by ADP, thrombin, and ristocetin.

Concentration[a]	Cell Means								
	ADP			Thrombin			Ristocetin		
	NaCl	SGS	$\bar{X}$	NaCl	SGS	$\bar{X}$	NaCl	SGS	$\bar{X}$
1	85.0	64.2	74.6	21.3	4.8	13.1	86.7	87.7	87.2
2	81.8	13.3	47.6	54.7	12.3	33.5	89.7	95.3	92.5
3	16.3	13.8	15.1	21.0	15.8	18.4	87.0	83.2	85.1
$\bar{X}$	61.0	30.0		32.3	11.0		87.8	88.7	

Source of variation	DF	MS	F	MS	F	MS	F
Trts	1	4216.7	50.3 ***	2048.0	22.6 ***	4.0	0.25
Conc'n	2	5325.5	64.5 ***	672.8	7.4 **	87.8	5.5 *
Trts x conc'n	2	1741.1	20.8 ***	544.3	6.0 *	33.8	2.1
Error + replicates	12	83.9		90.6		15.8	
Total	17						

* = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.001$

[a] No. representing concentration is arbitrary. Lowest number represents highest concentration. For further details, see methods.

Table B4. ANOVA results of platelet aggregation slopes induced by ADP, thrombin, and ristocetin.

		Cell Means								
		ADP			Thrombin			Ristocetin		
Concentration[a]		NaCl	SGS	$\bar{X}$	NaCl	SGS	$\bar{X}$	NaCl	SGS	$\bar{X}$
1		80.1	73.3	76.7	50.1	3.5	26.8	77.4	84.2	80.8
2		71.3	45.7	58.5	87.9	8.3	48.1	64.5	71.0	67.8
3		37.2	31.8	32.4	119.5	15.9	67.7	42.2	30.2	36.2
$\bar{X}$		61.4	50.3		85.8	9.3		52.8	26.5	
Source of variation	DF	MS		F	MS		F	MS		F
Trts	1	558.9		8.6 *	26,384.0		231.5 ***	0.8		0.01
Conc'n	2	2983.8		46.1 ***	2,511.5		22.0 ***	3159.9		49.9 ***
Trts x conc'n	2	248.5		3.8	1,230.8		10.8	173.9		2.7
Error + replicates	12	64.8			113.9			63.3		
Total	17									

* = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.001$

[a] No. representing concentration is arbitrary. Lowest number represents highest concentration. For further details, see methods.

Appendix C

APPENDIX OF CHAPTER 3

Table C1. Fly blood-meal weights from the right ear and back of previously-exposed and naive rabbits.

Variable and category	N	Mean (mg)	S.D. (mg)
For entire population	493	24.34	9.001
Naive rabbits (4)	248	24.26	8.922
Right ear	118	25.54	7.896
Female flies	59	27.32	8.594
Male flies	59	23.76	6.742
Back	130	23.10	9.645
Female flies	74	24.66	9.485
Male flies	56	21.05	9.552
Exposed rabbits (3)	180	24.75	9.213
Right ear	89	25.81	7.886
Female flies	43	29.08	8.410
Male flies	46	22.75	5.892
Back	91	23.72	10.287
Female flies	48	25.54	10.808
Male flies	43	21.69	9.382
Rabbit 3PD10[a]	65	23.49	8.857
Right ear	28	23.89	7.736
Female flies	15	27.50	7.244
Male flies	13	19.73	6.191
Back	37	23.18	9.714
Female flies	25	23.40	10.953
Male flies	12	22.75	6.839

[a] Rabbit 3PD10 was previously-exposed to the left ear and back, but never to the right ear.

( ) = no. of rabbits in the group

S.D. = standard deviation

Table C2. Fly blood-meal weights from the left (previously-exposed) and right (naive) ears of rabbits.

Variable and category	N	Mean (mg)	S.D. (mg)
Rabbits 5HB2, 3, 5, 6	348	25.59	7.384
Left ear	174	25.31	7.649
Female flies	95	28.43	8.025
Male flies	79	21.56	5.099
Right ear	174	25.87	7.121
Female flies	95	28.71	7.411
Male flies	79	22.46	4.968
Rabbit 3PD10	94	22.48	9.094
Left ear	51	23.21	9.178
Female flies	32	25.66	9.269
Male flies	19	19.08	7.572
Right ear	43	21.61	9.025
Female flies	25	23.32	10.009
Male flies	18	19.24	7.039

S.D. = standard deviation

Table C3. Probing frequencies of teneral tsetses fed on the back and ears of previously-exposed and naive rabbits.

Variable and category	N	Mean[a]	S.D.
Naive rabbits (4) - right ear	79	1.682	0.502
Female flies	40	1.615	0.561
Male flies	39	1.750	0.430
Exposed rabbits 3PD8 - 5FA4 right ear	47	1.605	0.457
Female flies	26	1.471	0.316
Male flies	21	1.771	0.551
Entire right ear population	126	1.653	0.485
Naive rabbits (3)	188	2.126	0.936
Back	101	2.505	1.068
Female flies	50	2.491	0.966
Male flies	51	2.518	1.169
Left ear	87	1.687	0.426
Female flies	48	1.563	0.240
Male flies	39	1.839	0.608
Exposed rabbits - 3PD8, 5FA4, 3PD10	107	2.142	0.954
Back	53	2.620	1.107
Female flies	29	2.201	0.846
Male flies	24	3.127	1.187
Left ear	54	1.673	0.407
Female flies	31	1.651	0.362
Male flies	23	1.701	0.476
Entire left ear and back population	295	2.132	0.941

[a] Probes = $\sqrt{\text{Probes} + 0.5}$ to normalize distribution (Snedecor and Cochran, 1967). Since 3PD10 was previously exposed to the left ear and shaved back but never the right ear, it was grouped with naive rabbits for analysis of the right ear, and then placed with previously exposed rabbits for analysis of the left ear and back.

Appendix D

APPENDIX OF CHAPTER 4

Table D1. Mean pupal weights of tsetses used in Experiment I (tsetse parameters affected by host-resistance).

	Rabbit(s)						
Larviposition	Control (naive)			Experimental (prev. exposed)			Student's
cycle (9 day)	N[a]	Mean (mg)	S.D.	N[a]	Mean (mg)	S.D.	t
1	106	25.93	3.78	58	25.61	4.06	0.513
2	85	28.37	4.09	58	27.24	3.89	1.683
3	89	30.06	4.07	72	28.11	4.64	2.829 *
4	75	30.52	3.74	59	29.96	4.35	0.105
5	72	29.91	4.40	46	27.70	4.57	2.419 *

Level of significance * $P < 0.05$

[a] Pupae under 20 mg weight are included in the above analysis. Removing pupae less than 20 mg did not alter the 0.05 significance levels of the Student's t values.

S.D. = standard deviation (mg).

Table D2. Female productivity of flies used in Experiment I (tsetse parameters affected by host-resistance).

YR - flies fed on the 3 naive young rabbits. PD9 - flies fed on 1 previously-exposed rabbit.

Larviposition cycle (9 day)	No. of female[a] flies		No. of pupae over 20 mg		No. of pupae under 20 mg		Percent of pupae under 20 mg		Female[b] productivity	
	YR	PD9	YR	PD9	YR	PD9	YR	PD9	YR	PD9
1	117	110	103	54	3	6	2.8	10.0	0.880	0.491
2	116	103	84	56	2	1	2.3	1.8	0.724	0.544
3	116	97	87	68	2	4	2.2	5.6	0.750	0.701
4	108	88	73	58	2	1	2.7	1.7	0.676	0.659
5	107	78	69	44	3	2	4.2	4.3	0.645	0.564
Total			416	280	12	14	2.9	5.0	x= 0.737	0.588

---

[a] No. of female flies was obtained from the mid-point percent survival of mortality curves for each cycle.

[b] Female productivity was determined using only those pupae over 20 mg weight.

Table D3. Mean pupal weights of flies used in Experiment II (tsetse parameters affected by host-resistance). YR = flies fed on 6 naive young rabbits. 3PD10 = flies fed on naive rabbit 3PD10. 3PD1 = flies fed on previously-exposed rabbit 3PD1.

Larviposition cycle (9 day)	Fly group	No. of pupae [a]	Mean pupal weight (mg)	S.D. (mg)
1	YR	71	27.88	3.54
	3PD10	56	28.68	4.54
	3PD1	58	27.03	4.61
2	YR	66	29.96	3.78
	3PD10	65	28.60	6.03
	3PD1	47	28.75	4.26
3	YR	61	30.00	5.03
	3PD10	63	29.21	5.69
	3PD1	39	27.49	7.58
4	YR	64	28.03	8.21
	3PD10	55	30.51	5.21
	3PD1	42	26.19	9.05
5	YR	51	30.74	4.45
	3PD10	47	30.60	7.03
	3PD1	33	27.56	6.13

[a] No. of pupae includes those pupae under 20 mg weight.

Table D4. Female productivity of flies used in Experiment II (tsetse parameters affected by host-resistance). Populations of flies were fed on a group of 6 young rabbits (YR), a single naive rabbit (3PD10), or a previously-exposed rabbit (3PD1).

Larviposition cycle (9 day)	Rabbit used	No. female[a] flies	No. pupae over 20 mg weight	Pupae under 20 mg		Female productivity
				N	Percent	
1	YR	100	68	3	4.2	0.680
	3PD10	108	57	2	3.4	0.528
	3PD1	88	57	3	5.0	0.651
2	YR	100	64	2	3.0	0.640
	3PD10	103	60	5	7.7	0.580
	3PD1	84	45	2	4.3	0.534
3	YR	100	62	1	1.6	0.622
	3PD10	100	61	3	4.7	0.610
	3PD1	73	36	3	7.7	0.495
4	YR	90	60	5	7.7	0.667
	3PD10	92	53	2	3.6	0.577
	3PD1	64	39	6	13.3	0.606
5	YR	88	49	2	3.9	0.560
	3PD10	83	46	2	4.2	0.556
	3PD1	55	31	3	8.8	0.562
All 5 cycles	YR	122-80	303	13	4.1	
	3PD10	120-74	277	14	4.8	
	3PD1	121-50	208	17	7.6	

[a] No. of female flies was calculated from the mid-point percent survival curves during each larviposition cycle.

Table D5. Pupal weights of flies used in Experiment III (local or systemic resistance). Rabbits 5HB5 and 5HB6 had the least difference in fly meal weights between their previously-exposed (left) and naive (right) ears. Rabbits 3PD10 and 5HB3 had the greatest difference.

Larviposition cycle (9 day)	Ear used	No. pupae[a]	Pupal weight mean (mg)	S.D.	Student's t
Flies fed upon rabbits 5HB5 and 5 HB6:					
1	naive	30	31.17	2.29	0.65
	exposed	36	30.71	3.27	
2	naive	33	32.60	2.55	0.68
	exposed	33	32.08	3.52	
3	naive	36	33.26	2.78	1.74
	exposed	40	32.06	3.18	
4	naive	38	33.24	3.27	3.27 *
	exposed	36	31.03	3.13	
5	naive	24	34.01	2.37	2.22 *
	exposed	25	32.41	3.08	
Flies fed upon rabbits 3PD10 and 5HB3					
1	naive	63	30.90	3.24	3.61 *
	exposed	68	28.84	3.22	
2	naive	58	31.69	2.73	1.33
	exposed	72	30.92	3.82	
3	naive	72	33.25	3.02	3.24 *
	exposed	65	31.38	3.61	
4	naive	61	32.67	2.73	2.87 *
	exposed	59	30.64	4.67	
5	naive	63	33.99	2.24	4.72 *
	exposed	49	31.04	3.86	

Level of significance * $P < 0.05$

[a] Analysis includes those pupae less than 20 mg weight.

Table D6. Female productivity of flies used in Experiment III (local or systemic resistance). Naive = flies fed on the naive ear of each pair of rabbits. Exp. = flies fed on the previously-exposed ear of each pair of rabbits.

Larviposition cycle (9 day)	No. female flies fed on each ear (from mortality curves)		No. pupae over 20 mg produced by flies on either ear		No. pupae under 20 mg produced by flies on either ear		Pupae under 20 mg on each ear (%)		Female productivity	
	Naive	Exp	Naive	Exp	Naive	Exp	Naive	Exp	Naive	Exp
Flies fed on rabbits 5HB5 and 5HB6 (rabbits with least difference in fly meal weights between ears):										
1	52	51	30	36	0	0	0	0	0.577	0.706
2	52	51	33	33	0	0	0	0	0.634	0.647
3	52	51	37	40	0	0	0	0	0.711	0.784
4	52	51	39	36	0	0	0	0	0.750	0.706
5	52	51	24	35	0	0	0	0	0.461	0.686
Flies fed on rabbits 3PD10 and 5HB3 (rabbits with greatest difference in fly meal weights between ears):										
1	86	80	64	69	0	0	0	0	0.744	0.862
2	86	78	59	72	0	1	0	1.4	0.682	0.923
3	86	78	73	65	1	1	1.4	1.5	0.847	0.833
4	84	77	62	60	0	3	0	4.8	0.738	0.779
5	84	77	65	51	1	3	1.5	5.6	0.774	0.663

Table D7. Mean pupal weights of flies used in Experiments IV and V (effect of naturally produced antibodies).

Experiment IV:

Va = flies fed on 6 naive rabbits. Ab = flies fed on 4 prev-exp rabbits.

Larviposition	Va flies			Ab flies			Student's
cycle (9 day)	N	mean	S.D.	N	mean	S.D.	t
1	106	30.99	3.15	97	28.81	2.68	5.299 *
2	109	33.93	3.02	94	30.88	2.92	7.297 *
3	97	34.03	3.31	88	32.56	3.30	3.018 *
4	84	34.10	4.64	83	32.88	3.10	1.992 *
5	65	34.30	3.97	63	32.72	3.45	2.392 *

Experiment V:

HC = flies fed on 5 naive rabbits, bled until haematocrits were similar to rabbits used to feed Ax flies. Ax = flies fed on 4 previously-exposed rabbits made anemic by excessive tsetse feeding.

Larviposition	HC flies			Ax flies			Student's
cycle (9 day)	N	mean	S.D.	N	mean	S.D.	t
1	53	29.99	3.00	52	30.56	2.78	1.012
2	52	31.40	3.45	54	32.48	2.82	1.444
3	45	32.64	2.95	46	31.78	2.81	1.410
4	38	32.17	4.08	42	31.89	2.99	0.347
5	36	34.24	3.14	34	32.56	3.79	2.023 *

Level of significance * $P < 0.05$

Analysis includes pupae less than 20 mg weight.

Table D8. Female productivity of flies used in Experiments IV and V (effect of naturally produced antibodies). Analysis includes only those pupae over 20 mg weight.

Experiment IV: Va = flies fed on 6 naive rabbits. Ab = flies fed on 4 previously-exposed rabbits.

Larviposition cycle (9 day)	No. female flies		No. pupae over 20 mg		No. pupae under 20 mg		Percent of pupae under 20 mg		Female productivity	
	Va	Ab	Va	Ab	Va	Ab	Va	Ab	Va	Ab
1	118	117	104	97	1	0	1	0	0.881	0.829
2	115	115	109	94	0	0	0	0	0.949	0.814
3	114	109	97	86	0	3	0	3.4	0.854	0.788
4	111	107	84	81	1	3	1.1	3.6	0.757	0.754
5	103	105	64	63	1	0	1.6	0	0.619	0.600
Total			463	421	3	6	0.6	1.4		

Experiment V: HC = flies fed on 5 naive rabbits, bled until haematocrits were similar to rabbits used to feed Ax flies. Ax = flies fed on 4 previously-exposed rabbits made anemic by excessive tsetse feeding.

Larviposition cycle (9 day)	HC	Ax	HC	Ax	HC	Ax	HC	Ax	HC	Ax
1	58	60	51	53	1	0	2.0	0	0.879	0.883
2	57	59	53	52	1	2	1.9	3.7	0.929	0.881
3	56	59	45	46	0	0	0	0	0.803	0.779
4	53	58	37	40	0	1	0	2.4	0.698	0.690
5	49	54	36	34	0	0	0	0	0.735	0.630
Total			222	225	2	3	0.9	1.3		

AUTOBIOGRAPHICAL SKETCH

I was born in Edmonton in 1953, and remained there until completion of grade 1. Following my father's transfer to Toronto, I spent grades 2 and 3 in Clarkson, Ontario. A similar transfer in 1962 resulted in our moving to Brandon, Manitoba, where I spent from grades 4 to 12. With interests in fishing, hunting and trapping, I became enthusiastic about nature. Despite this fact, I never took biology in high school so that my choice of courses at university would not be restricted. As a student, my high school averages ranged between 67 and 72. Unlike many students with an interest in biology, my best marks were in literature and composition and my troubles in French. My high school summers were spent working at manual labour. I received my grade 12 diploma in June, 1971.

Undecided about my choice of education at university, and unprepared for the high school-university switch, I made the mistake of enrolling in pre-med courses. Second year, I majored in Zoology, with my interests repaying themselves in grades. My first two summers during university life were spent on experience, not money. Working for Dr. D.B. Stewart, professor of Zoology at Brandon University, and funded by an NRC grant, I spent time capturing, banding and removing blood samples from migratory birds in a study of the prevalence of a bacteria. In the spring of 1974, I graduated with a 3 yr B.Sc. from Brandon University, majoring in Zoology and minoring in Botany. At university, the lectures of Dr. J.A. MacLeod, in parasitology and medical and veterinary entomology, had interested me most. Being young, and in a competitive position with many other graduates, I decided to pursue the field of research for personal achievement and not as a job prospect.

Following my interests to examine host-parasite interaction, I was advised to pursue Entomology rather than Zoology because of competition, research funding, and job opportunities. Further advice directed me to the Department of Entomology, University of Alberta, and specifically to Dr. R.H. Gooding. Accepted as a qualifying graduate student, I arrived in Edmonton in the fall of 1974 after spending the summer in Manitoba, working as a temporary biologist for Ducks Unlimited of Canada. With unsettled decisions on a research proposal, although adequate choices and funding available through Dr. Gooding, I chose to work with the interactions between tsetses and rabbits as a host/biting-fly model. Since research could be done year round and a number of parameters could be adequately controlled, the choice proved advantageous. This thesis is a result of 3 efforts. First, it is a result of my interest in the field. Second, it is the result of an integrative rather than a specific approach to a complex problem. Third, it is a way of repaying my advisor for the help he has given me.